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# Thyroid receptor antagonism as a contributory mechanism for adipogenesis induced by environmental mixtures in 3T3-L1 cells

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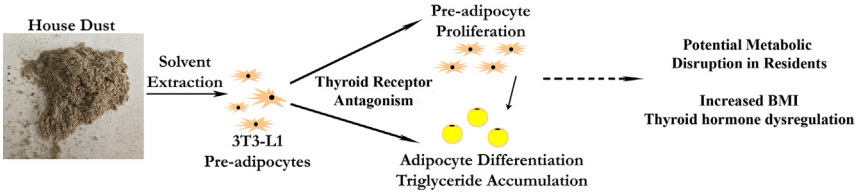
## HIGHLIGHTS

- Tested ability of indoor house dust extracts (HDE) to promote adipogenesis *in vitro*.
- The majority of HDE promoted triglyceride accumulation and pre-adipocyte proliferation.
- Adipogenic activity was positively correlated with each of twelve flame retardants.
- Adipogenic activity was mediated in part by thyroid receptor antagonism of HDE.
- Adipogenic activity of HDE was associated with metabolic health of residents.

## GRAPHICAL ABSTRACT

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## ABSTRACT

We previously demonstrated that indoor house dust extracts could induce adipogenesis in pre-adipocytes, suggesting a potential role for indoor contaminant mixtures in metabolic health. Herein, we investigated the potential role of thyroid receptor beta (TRβ) antagonism in adipogenic effects (dust-induced triglyceride accumulation and pre-adipocyte proliferation) following exposure to environmental mixtures (indoor house dust extracts). Concentrations of specific flame retardants were measured in extracts, and metabolic health information was collected from residents (n = 137). 90% of dust extracts exhibited significant adipogenic activity, >60% *via* triglyceride accumulation, and >70% *via* pre-adipocyte proliferation. Triglyceride accumulation was positively correlated with concentrations of each of twelve flame retardants, despite most being independently inactive; this suggests a putative role for co-exposures or mixtures. We further reported a positive correlation between dust-induced triglyceride accumulation and serum thyroid stimulating hormone concentrations, negative correlations with serum free triiodothyronine and thyroxine concentrations, and a positive and significant association between dust-induced triglyceride accumulation and residents' body mass index (BMI). We hypothesized that inhibition of TR antagonism might counteract these effects, and both addition of a TR agonist and siRNA knock-down of TR resulted in decreased dust-induced triglyceride accumulation in a subset of samples, bolstering this as a contributory mechanism. These results highlight a contributory role of environmental TR antagonism as a putative factor in metabolic health, suggesting that further research should evaluate this mechanism and determine whether *in vitro* adipogenic activity could have utility as a biomarker for metabolic health in residents.


## 1. Introduction

Increasing research has suggested a role for endocrine disrupting chemicals (EDCs) in the rising rates of obesity and associated metabolic

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diseases. EDCs include 1000 or more synthetic or naturally occurring chemicals or mixtures that can interfere with any aspect of hormone action (The Endocrine Disruption Exchange (TEDX), 2018); some of these, termed "metabolic disruptors", can increase weight gain or contribute to metabolic dysfunction in animal models and/or promote triglyceride accumulation *in vitro* (Heindel et al., 2015; Janesick and Blumberg, 2016). Confirming putative metabolic disruptors *in vivo* is cost and time-prohibitive. As such, utilizing appropriate *in vitro* models is crucial for screening individual environmental chemicals and characterizing mixtures, due to ease of assessing multiple chemicals, concentrations, and co-exposures for much lower cost and time investments. The committed 3T3-L1 mouse pre-adipocyte cell line is commonly used for this purpose; following exposure to adipogenic chemicals, these cells differentiate into adipocytes, undergo morphological changes, accumulate triglycerides, and eventually develop into a mature white fat cell (Green and Kehinde, 1975; Green and Meuth, 1974). This model and others have been rigorously applied to characterize putative metabolic disruptors over the last forty years, with an increasing number of publications demonstrating effects on adipocyte commitment, differentiation, proliferation, and lipid accumulation (reviewed in (Heindel et al., 2015; Heindel et al., 2017). Many of these adipogenic chemicals have been further demonstrated to be active *in vivo* (Angle et al., 2013; Chamorro-Garcia et al., 2013; Li et al., 2011; Masuno et al., 2005), bolstering the reliability and translational capability of this model. Moreover, the molecular mechanisms promoting adipocyte development are highly conserved across vertebrate species (Fu et al., 2005; Zhao et al., 2015), substantiating the utility of this model in predicting potential human metabolic concerns. Modulation of numerous molecular pathways can regulate adipogenesis, including activation of the peroxisome proliferator-activated receptor-gamma (PPARγ), liver X receptor (LXR), glucocorticoid receptor (GR), retinoid X receptor (RXR), and/or antagonism of the thyroid hormone receptor (TR), farnesoid X receptor (FXR), androgen receptor (AR), and others (Fu et al., 2005; Niemelä et al., n.d.; Kassotis et al., 2017a). EDCs that can modulate these pathways are ubiquitous, being found in numerous consumer products (furniture, electronics, plastics, food packaging, *etc.*) and accumulating in the outdoor and indoor environment (Fraser et al., 2012; Hoffman et al., 2015a; Rudel et al., 2010; Watkins et al., 2011). As a result, humans are exposed to complex mixtures of EDCs throughout development, with exposure beginning during gestation (Dallaire et al., 2003; Houlihan et al., n.d.) and continuing through lactation (Landrigan et al., 2002; Mogensen et al., 2015) and from the indoor environment throughout their lives (Fraser et al., 2012; U.S. Environmental Protection Agency (EPA), 2017; Rudel et al., 2003; Watkins et al., 2013).

Research by our laboratory and others has demonstrated that indoor house dust is consistently contaminated with numerous semi-volatile organic contaminants (SVOCs) globally, many that are suspected of being hormonally active, including phthalates, flame-retardants, pesticides, parabens, and perfluoroalkyl substances (PFASs) (Rudel et al., 2003; Stapleton et al., 2006; Stapleton et al., 2009; Rasmussen et al., 2013; Mitro et al., 2016; Kademoglou et al., 2017; Suzuki et al., 2013). The EPA estimates that from birth through six years of age, children ingest approximately between 60 and 100 mg of dust per day from indoor environments (U.S. Environmental Protection Agency (EPA), 2017), contributing to chronic oral and inhalation exposures to EDCs present in the dust (Fraser et al., 2012; Rudel et al., 2003; Watkins et al., 2013). Furthermore, our laboratory and others have previously demonstrated that levels of various classes of contaminants in household dust are bio-accessible and highly correlated with blood and urine levels in residents (Hoffman et al., 2015a; Watkins et al., 2011; Fang and Stapleton, 2014; Phillips et al., 2018; Pawar et al., 2016; Lim et al., 2014; Meeker et al., 2013; Stapleton et al., 2012; Johnson et al., 2010), suggesting a clear exposure pathway. Due to the diversity of potentially endocrine-active chemicals in house dust, several laboratories have assessed various nuclear receptor bioactivities, reporting agonist activities for PPARγ, GR, and ER, and antagonist activities for AR and TR, at concentrations between 10 and 40 μg dust equivalence (Suzuki et al., 2013; Chou et al., 2015; Suzuki et al., 2007; Kollitz et al., 2018). Previous research from our laboratory assessed the ligand binding and subsequent activation of PPARγ by house dust extracts; 84% exhibited significant PPARγ binding at ≥120 μg dust equivalence per well (Fang et al., 2015a), and 60% exhibited significant receptor activation at ≥4 μg dust per well (Fang et al., 2015b; Fang et al., 2015c). More recently, we profiled the adipogenic activity (defined as the ability to stimulate triglyceride accumulation and/or pre-adipocyte proliferation) of 40+ SVOCs common in indoor house dust samples (Kassotis et al., 2017b); more than two thirds of these chemicals exhibited significant adipogenic activity. We further assessed a small subset of 11 house dust extracts, reporting that ten of 11 exhibited significant adipogenic activity at <20 μg dust per well (Kassotis et al., 2017b). This suggests a potential role of indoor house dust in resident's metabolic health, as well as a need to determine molecular mechanisms promoting these effects, causative chemicals present in these mixtures, and assessment of whether the adipogenic activities in dust are associated with adverse metabolic health in residents living in these homes.

Specifically, antagonism of TR has been demonstrated to robustly modulate adipocyte differentiation, purportedly through reciprocal regulation with PPARγ (Lu and Cheng, 2010). We previously demonstrated that antagonism of TR was one of the most active/potent adipogenic pathways, as measured *via* triglyceride accumulation in 3T3-L1 pre-adipocytes (Kassotis et al., 2017a), suggesting a potential role in disruption of TR in environmental mixture-induced differentiation. Thyroid hormones have long been considered anti-obesogenic, and hypothyroid-associated adiposity can be reduced with supplementation (Bryzgalova et al., 2008). All of the TR isoforms are expressed in white and brown adipocytes, with TRα purportedly playing a larger role in regulating thermogenesis and TRβ in cholesterol metabolism and lipogenesis, as well as directly regulating (or through crosstalk with PPARγ) a number of genes and enzymes necessary for pre-adipocyte proliferation and adipocyte differentiation (Obregon, 2008). As such, a parallel study examined the extent of TRβ antagonism in the same dust extracts used in this study (Kollitz et al., 2018), and these data were assessed as a potential contributory mechanism in the observed adipogenic effects. Approximately half of these extracts significantly antagonized TRβ activity, decreasing triiodothyronine (T3)-mediated efficacy by 20–67% compared to the T3 added agonist control. Further, potencies (concentrations required to inhibit 20% of T3-mediated efficacy) were negatively correlated with free serum thyroxine (FT4) concentrations in residents (more potent samples exhibited greater FT4 concentrations), suggesting a proximate impact of the environmental TR ligands on thyroid function in these individuals.

The goals of this study were to more comprehensively assess the metabolic disruption potential of indoor house dust extracts and further assess the role that TRβ plays in observed adipogenesis. Murine 3T3-L1 cells (Zenbio, Inc.) were used to screen and evaluate the dose responses of 137 indoor house dust extracts for triglyceride accumulation and pre-adipocyte proliferation. Twelve flame retardants (FRs) were previously measured in these dust extracts (Hoffman et al., 2017a); herein, we performed correlations to determine whether FR concentrations were associated with the observed activities. Further, the observed adipogenic activities were examined for their associations with health outcomes in the adult residents of the households where dust was collected. Health measurements included body mass index (BMI), serum thyroid stimulating hormone (TSH), serum free triiodothyronine (FT3), and FT4. We hypothesized that house dust would promote triglyceride accumulation and pre-adipocyte proliferation at low concentrations, and that this would be mediated in part by inhibition of TRβ.

## 2. Materials and methods

### 2.1. Chemicals

Chemicals were purchased as follows: rosiglitazone (Sigma cat # R2408, ≥ 98%), 1–850 (Millipore cat # 609315, ≥ 98%), and triiodothyronine (T3; VWR cat # 80057–656, ≥ 98%). Stock solutions were prepared in 100% DMSO (Sigma cat # D2650) and stored at −20 °C between uses.

### 2.2. Patient enrollment and clinical assessments

House dust samples (n = 137) were collected and processed as described previously (Hoffman et al., 2017a). Briefly, dust samples were obtained from a case-control study investigating indoor exposures and thyroid cancer. Patients newly diagnosed with papillary thyroid cancer between April 2014 and January 2016 and referred to endocrinology or endocrine surgery at the Duke Cancer Institute or Duke University Hospital were approached by their physician. Willing participants were contacted by the study team and enrolled, and control participants were recruited from other Duke patients undergoing routine wellness care or care for unrelated medical issues or were recruited from by posted notices in the Durham, NC area. Inclusion was restricted to individuals living within 50 miles of Duke University (Durham, NC, USA) and to individuals who had lived in the same home for at least two years to ensure that levels of exposure were reflective of historical exposure occurring over the last several years. Control inclusion was restricted to individuals without a previous diagnosis of malignant or benign thyroid disease, and pregnant women were excluded, as their thyroid hormone levels would be expected to be different than a non-pregnant population. Upon enrollment, researchers visited each home to obtain house dust samples and collect information using a study questionnaire, including self-reported age, race/ethnicity, educational attainment, height and weight (for calculation of BMI), time lived in current residence, and other general health information. Laboratory measurements of FT3, FT4, and TSH were performed for all controls, but not for cancer patients, as their thyroid hormones are dysregulated and, in many cases, regulated with synthetic thyroid hormones administered by their medical team. Thyroid hormones were quantified by LabCorp (Burlington, NC) following standard protocols (Hoffman et al., 2017a). All study protocols were reviewed and approved by the Duke University Health System Institutional Review Board.

### 2.3. Dust sample collection and processing

House dust samples (n = 137) were collected and processed as described previously (Hoffman et al., 2017a). Samples were collected from households between 2014 and 2016 from residents that had lived in their households for at least two years and who were instructed not to vacuum for at least two days prior to collection. During collection, the main living area of the home was vacuumed (area in which the participant reported spending the most time), and dust was collected in a cellulose thimble (Stapleton et al., 2012; Stapleton et al., 2014), wrapped in foil, and stored at −20 °C. To process, samples were sieved to <500 μm, approximately 100 mg of dust was extracted with 50:50 dichloromethane:hexane (used to extract a wide range of chemical classes) *via* sonication, and extracts were concentrated under nitrogen gas. Half of each extract's volume was purified using solid-phase extraction cartridges, and used for targeted analysis of brominated and organophosphate flame retardants (BFRs and PFRs, respectively) *via* GC/MS as described previously (Hoffman et al., 2017a). The second half of this fraction was evaporated to dryness and reconstituted in 100 μL DMSO for use in bioassays. Laboratory blanks were prepared using laboratory solvents and techniques in the absence of dust to ensure that lab procedures did not impart any active chemicals to our assays. None of these samples exhibited significant triglyceride accumulation or pre-adipocyte proliferation at any concentration tested (Fig. S1).

### 2.4. 3T3-L1 cell care and differentiation and outcome measurements

3T3-L1 cells (Zenbio cat# SP-L1-F, lot# 3T3062104; Research Triangle Park, NC) were maintained as described previously in pre-adipocyte media (Dulbecco's Modified Eagle Medium – High Glucose; DMEM-HG; Gibco cat# 11995, supplemented with 10% bovine calf serum and 1% penicillin and streptomycin; Gibco cat# 15140) (Kassotis et al., 2017a). Cells were utilized between passages 8 (at purchase) and 14, maintained in a sub-confluent state until differentiation. Positive and negative controls were included in each assay plate, with no significant changes in control responses observed across time.

3T3-L1 cells were induced to differentiate as described in detail previously (Kassotis et al., 2017a; Kassotis et al., 2017b). Briefly, cells were seeded in pre-adipocyte media into 96-well tissue culture plates (Greiner cat # 655090) at ~30,000 cells per well. Once confluent, cells were allowed 48 h for growth arrest and to allow initiation of clonal expansion. Media was then replaced with test chemicals and/or controls using a 0.1% DMSO vehicle in differentiation media (DMEM-HG with 10% fetal bovine serum, 1% penicillin/streptomycin, 1.0 μg/mL human insulin, and 0.5 mM 3-isobutyl-1-methylxanthine, IBMX). After 48 h of differentiation induction, media was replaced with fresh dilutions of test chemicals in adipocyte maintenance media (differentiation media without IBMX), and treatments of media and test chemicals were refreshed every 2–3 days until assay, ten days after induction.

### 2.5. 3T3-L1 triglyceride accumulation, cell proliferation, and cell viability measurements

Plates were assayed for triglyceride accumulation, DNA content, and cell viability after ten days of differentiation as described in great detail previously (Kassotis et al., 2017a; Kassotis et al., 2017b). Briefly, media was removed and cells rinsed with Dulbecco's phosphate-buffered saline (DPBS; Gibco cat # 14040) before replacing with 200 μL of a dye mixture (19 mL DPBS, 1 drop/mL NucBlue® Live ReadyProbes® Reagent (cell proliferation/cytotoxicity measure of DNA content; Thermo cat # R37605) and 500 μL AdipoRed™ (intracellular lipid measure of triglyceride accumulation; Lonza cat # PT-7009) per plate). Plates were protected from light and incubated at room temperature for approximately forty minutes; then fluorescence was measured with excitation 485 nm/emission 572 nm for AdipoRed™, 360/460 for NucBlue®. Following the lipid and DNA readings, cell viability was assessed using the CellTiter-Glo™ (cell viability measure *via* ATP content; Promega

**Table 1**
Descriptive results of adipogenic activity across dust extracts.

| Dust sample metrics | Range | Median | Mean |
|---|---|---|---|
| Dust extract concentrations (μg/mL) | | | |
| Overall | 10.1–4449 | 494.6 | 589.2 |
| Lower tertile | 10.1–283.7 | 149.3 | 152.7 |
| Middle tertile | 290.4–626.7 | 494.6 | 469.7 |
| Upper tertile | 629.9–4449 | 990.2 | 1145 |
| Dust-induced triglyceride accumulation (%) | | | |
| Overall | 0–388.9% | 14.9% | 37.4% |
| Lower tertile | 0–109.5% | 2.2% | 13.9% |
| Middle tertile | 0–388.9% | 27.2% | 44.8% |
| Upper tertile | 0–208.7% | 43.3% | 54.0% |
| Dust-induced cell proliferation (%) | | | |
| Overall | 0–88.7% | 12.2% | 16.0% |
| Lower tertile | 0–60.0% | 12.6% | 15.5% |
| Middle tertile | 0–65.5% | 10.4% | 14.9% |
| Upper tertile | 0–88.7% | 14.3% | 17.6% |

Descriptive statistics for dust extract concentrations and adipogenic activity metrics (both triglyceride accumulation and pre-adipocyte proliferation) for n = 137 samples. Tertiles defined based on dust extract concentrations across groups. Dust extract concentrations provided as highest test concentration; each extract was tested at its highest concentration and four two-fold dilutions thereof.

cat# G7572) assay as described previously (Kassotis et al., 2017b). Briefly, 170 μL of DPBS mixture was removed from all wells, and the remaining 30 μL was mixed with 30 μL of CellTiter-Glo™ reagent. Plates were incubated for ten minutes prior to reading luminescence. CellTiter-Glo™ viability was assessed as a percent change from differentiated DMSO controls. Inhibited cell health was assessed *via* deviations of ≥15% in either cell viability (ATP) and/or cytotoxicity (DNA content) assays, as we described previously in detail (Kassotis et al., 2017a; Kassotis et al., 2017b). Four technical (replicates within each assay plate) and three biological replicates (separate cell passages/assays) were utilized for every test chemical and concentration reported herein.

For adipogenic activity, percent activities (efficacies) across the tested dose response range were calculated relative to the maximal rosiglitazone-induced fold induction over intra-assay differentiated vehicle controls (0.1% DMSO), after correcting for background fluorescence. Rosiglitazone was utilized as the positive control herein due to selective, robust, and potent binding to PPARγ (Lehmann et al., 1995; Spiegelman, 1998; Seimandi et al., 2005); it is often utilized as a positive control in adipogenesis assays (Watkins et al., 2015; Chappell et al., 2018; Chamorro-Garcia et al., 2018; Foley et al., 2017; Janesick et al., 2016; Temkin et al., 2016; Zebisch et al., 2012), and allows for easier comparisons to be made in test chemical response across laboratories and studies. DNA content was calculated as percent change from vehicle controls for each chemical at each concentration and was then used to normalize total triglyceride values to obtain triglyceride content per unit DNA (as a proxy for triglyceride accumulation per cell). Both total triglyceride content and triglyceride normalized to DNA content are provided throughout to ensure comparability between studies, as many do not report DNA content/normalized triglycerides. Potencies were determined using $EC_{10}$ values (concentrations of each chemical/mixture that exhibit 10% of their own maximal activity) as determined using GraphPad Prism 7.0 (La Jolla, CA, USA). As we determined that extracted dust mass could be a potential confounder in maximal bioactivity, normalized metrics were created for further assessment: efficacy of response at 500 μg/mL dust extract concentration and potency (μg/mL extract concentration) at which 10% response was observed. Full dose responses of dust extracts were plotted and used to calculate the point where dose responses crossed the 500 μg/mL and 10% efficacy lines, respectively. Values were extrapolated as necessary for efficacy and potency values for samples approaching the cut-off; potency values

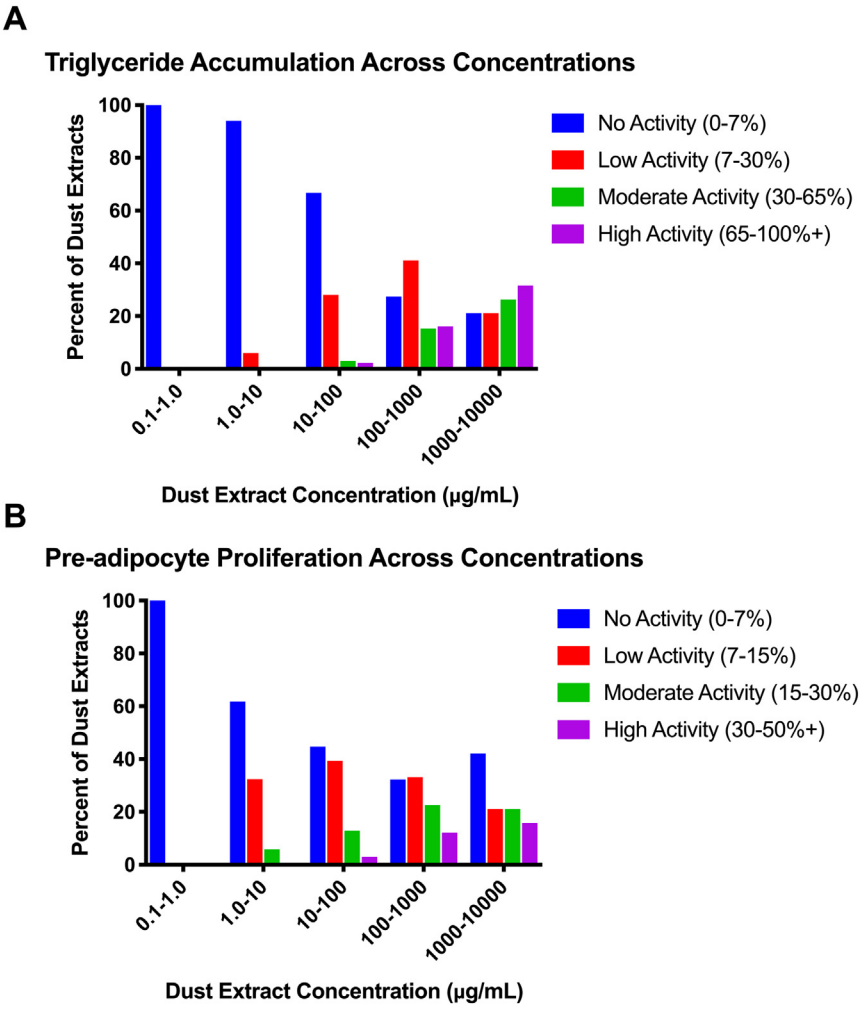


**Fig. 1.** Adipogenic activity of indoor house dust extracts varies by concentration. 3T3-L1 cells were induced to differentiate as described in Methods. Cells were assessed for degree of adipocyte differentiation after ten days of differentiation while exposed to dose responses (2-fold dilutions from maximal concentrations) of indoor house dust extracts. Dust samples exhibiting significant adipogenic activity relative to the differentiated vehicle control across each concentration range were separated according to efficacy. As such, the proportion of samples exhibiting significant activity in each concentration range can be calculated by summing the low, moderate, and high activity groups. (A) Distribution of normalized triglyceride accumulation per cell relative to maximal intra-assay response for rosiglitazone (normalized to DNA content) for each dust sample at every concentration tested. (B) Distribution of increased DNA content (pre-adipocyte proliferation) relative to a differentiated vehicle control for each dust sample at every concentration tested. Bars represent the percent of dust extracts that exhibited significant activity when tested at each concentration range. Sample number examined at each concentration range varied due to varying dust collected in each home: n = 1, 35, 132, 124, and 19 for 0.1–1.0, 1.0–10, 10–100, 100–1000, and 1000–10,000, respectively, for both (A) and (B).

were not extrapolated when there was no apparent activity (samples not approaching 10% activity), as potencies cannot be calculated for inactive chemicals/samples.

### 2.6. Ligand recovery and siRNA mechanism interrogation experiments

For ligand recovery experiments, nine house dust extracts were randomly selected for further interrogation of molecular mechanisms and demonstrated a range of TRβ bioactivities. Cells were differentiated as described above and cells co-exposed to dose responses of dust extracts (maximal concentrations of each and four 2-fold serial dilutions) and 10 μM T3, under the presumption that TRβ antagonism was a causative factor in the triglyceride accumulation, and thus a TRβ agonist should inhibit some of the dust-induced adipogenic activity. T3 co-treatment responses were compared to dust alone to determine the effect of TRβ agonism in "recovering" potential TRβ antagonism-mediated effects. Two concentrations are reported for each dust sample to demonstrate effects across the dose response for each sample; high-dose dust utilized the maximal dust concentration for each sample (1:1000 dilution of DMSO extract), which varied according to quantity of dust collected from homes, and low-dose dust utilized the lowest concentration in each dose response (16-fold dilutions of maximal test concentration), with comparisons made between concentrations and relative to controls (T3 without dust).

For siRNA experiments, the same nine dust extracts were selected, and the low concentration was tested for each (16-fold dilutions of maximal concentrations). Sub-confluent cells were seeded into three 96-well plates as described above. One plate was retained as a non-

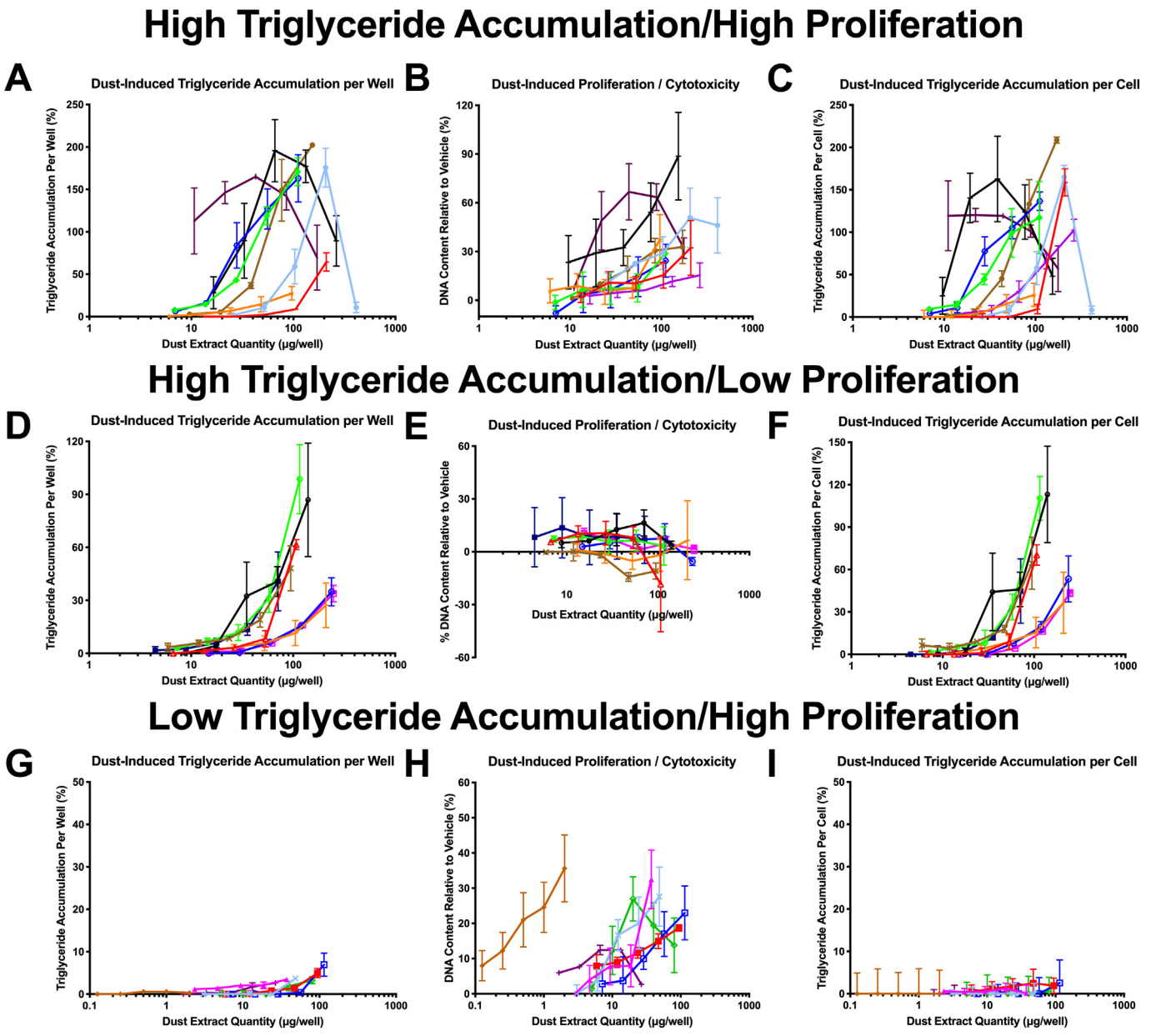


**Fig. 2.** Dust extracts induce heterogeneous activity across triglyceride accumulation and proliferation. 3T3-L1 cells were induced to differentiate as described in the Methods. Cells were exposed to dose-responses (2-fold dilutions from maximal concentrations) of indoor house dust extracts and assessed for degree of triglyceride accumulation and pre-adipocyte proliferation ten days post-exposure. (A, D, G) Percent raw triglyceride accumulation per well relative to maximal intra-assay response for rosiglitazone for select dust extract dose-responses. (B, E, H) Increase (pre-adipocyte proliferation) or decrease (potential cytotoxicity) in DNA content relative to vehicle control for select dust extract dose-responses. (C, F, I) Percent normalized triglyceride accumulation per cell relative to maximal intra-assay rosiglitazone response (normalized to DNA content) for select dust extract dose-responses. Data presented as mean ± SEM from three independent experiments (n = 4 per experiment). Colors represent dust samples from unique households.

transfected control, one was transfected with a negative control siRNA (Santa Cruz Biotech, SCBT, cat# sc-37,007), and the third with both TRα (SCBT cat# sc-36,708) and TRβ (SCBT cat# sc-38,891) siRNAs, using siRNA Transfection Reagent (SCBT cat# sc-29,528) according to modified manufacturer's instructions (Santa Cruz Biotechnology, 2017) and similarly to previously reported (Foley et al., 2017). Briefly, after seeding cells into 96-well plates, 8 μL siRNA and 64 μL transfection reagent were diluted in transfection media (SCBT cat# sc-36,868) and incubated at room temperature for 45 min. Following incubation, cells were rinsed with transfection medium, and 40 μL of the transfection mixture was applied to each well of the plate. Cells were transfected for approximately six hours before adding 160 μL pre-adipocyte maintenance media to end transfection and begin recovery. Cells were confluent by the following day and were then allowed a further two days to undergo growth arrest and revert to clonal expansion before cells were differentiated as described above and assessed for triglyceride accumulation and pre-adipocyte proliferation. Comparisons were made between siRNA-TR responses and both naïve and negative siRNA controls. Full dose responses of rosiglitazone, 1–850 (selected due to potent and selective TR antagonism (Schapira et al., 2003)), T3 (selected as an endogenous TR agonist), and varied cell-based controls (differentiated DMSO vehicle controls, undifferentiated controls, were examined with naïve, siRNA-null, and siRNA-TR cells to determine relative effects of these TR-modulating chemicals and their potential interplay with dust extracts and siRNA knock-downs. TR agonist and antagonist dose responses under these varying conditions were utilized to assess the degree of TR knockdown in the siRNA experiments. As reported previously with PPAR knock-down utilizing this procedure (Foley et al., 2015), our functional assessment of knock-down success revealed an approximate 67% reduction in TR antagonist (1–850) efficacy (Fig. S8).

### 2.7. Statistical analysis

Data are presented as means ± SEM from four technical replicates of three or four independent biological replicates (two for thyroid mechanism interrogation assays). Results were analyzed using a one-way ANOVA and Dunnett's post-hoc test. Differences between treatment and control groups were considered statistically significant at $p < 0.05$. $EC_{10}$ values were estimated using curves generated from raw fluorescence data using a 4-parameter variable-slope Hill model, utilizing the "log (Agonist) vs. response – Find ECanything" feature within the nonlinear regression function, and assigning an F value of 10 (for $EC_{10}$ calculation) and a bottom of 0. Relationships between measures of adipogenic activity and the concentrations of flame retardants in samples and adverse health outcomes in the residents were assessed using Spearman correlation analyses. To address potential joint effects of multiple compounds, we further conducted a principal components analysis. We identified three primary factors consisting of the summed polybrominated diphenyl ethers (other than BDE-209), the BFRs comprising Firemaster 550™ (TBB, TBPH), and the PFRs. Correlations with these factors were generally similar to individual components (Table S1) and did not provide additional insights, so we have opted to discuss only individual chemicals within the text. Associations between adipogenic activity and human health endpoints were first assessed using Spearman correlations. Robust regressions were also performed to assess relationships between adipogenic activity metrics and resident health outcomes (Hampel et al., 1986; Yohai, 1987). These models allowed for the control of potential confounding variables, including age, sex, race, and education, which we anticipate could be related to health outcomes as well as the types of contaminants in the home environment driving the measured adipogenic activity. Analyses were performed using GraphPad Prism 7.0 or SAS 9.4.

## 3. Results

Dose responses of 137 house dust extracts (1000-fold dilution into exposure media and four subsequent serial two-fold dilutions) were assessed for adipogenic activity utilizing 3T3-L1 cells. Cells were differentiated over ten days of treatment with extracts and then assessed for pre-adipocyte proliferation (DNA content, relative to vehicle control), triglyceride accumulation (total triglycerides per well and per cell, normalized to DNA content; both relative to maximal rosiglitazone response), and cell viability (ATP production).

### 3.1. Adipogenic activity of indoor house dust extracts

Given differing quantities of dust collected from each home, dust extract concentrations in contact with the cells varied from 10 to 4449 μg/mL (dust equivalent concentration; Table 1), and each was tested at its maximal concentration and at four two-fold dilutions thereof. The majority of dust extracts exhibited adipogenic activity in our assay system, with >60% promoting significant triglyceride accumulation of up to 389% relative to the rosiglitazone-induced maximum, >70% promoting significant pre-adipocyte proliferation of up to 89% relative to the differentiated vehicle control, and ~90% exhibiting one or both activities (Table 1). >40% of extracts exhibited effects at concentrations <10 μg dust/well (50 μg/mL extract test concentration), and approximately 10% of extracts exhibited neither triglyceride accumulation nor pre-adipocyte proliferation. Approximately 6% of samples tested between 1 and 10 μg/mL (in diluted extracts) exhibited significant triglyceride accumulation, relative to 38% exhibiting significant pre-adipocyte proliferation (Fig. 1). Adipogenic activity increased with dust extract concentration tested, with 33% of samples exhibiting significant triglyceride accumulation when diluted extracts were tested between 10 and 100 μg/mL, 73% when tested between 100 and 1000 μg/mL, and 79% when tested between 1000 and 10,000 μg/mL (Fig. 1A); notably, some samples exhibited reduced activity at their highest concentration due to apparent cytotoxicity. The efficacies of triglyceride accumulation induced by diluted extracts also increased with increasing concentration; when diluted extracts were tested at concentrations from 10 to 100 μg/mL, 28% of samples exhibited 7–30% triglyceride accumulation, 3% exhibited 30–65%, and 2% exhibited 65–100%+, compared to when tested at 100–1000 μg/mL, 41% exhibited 7–30%, 15% exhibited 30–65%, and 16% exhibited 65–100% + (Fig. 1A). This was mirrored but less pronounced with pre-adipocyte proliferation, with 55.3% of diluted samples exhibiting significant proliferation when tested at concentrations between 10 and 100 μg/mL, 68% when

**Table 2**
Correlations of adipogenic activity with chemical concentrations in dust extracts.

| Chemical | Total triglyceride accumulation (per well) | DNA content (pre-adipocyte proliferation) | Normalized triglyceride accumulation (per cell/DNA) |
|---|---|---|---|
| Brominated flame retardants (BFRs) | | | |
| BDE-47 | 0.268** | −0.096 | 0.279** |
| BDE-99 | 0.403** | −0.124 | 0.330** |
| BDE-100 | 0.321** | −0.043 | 0.339** |
| BDE-153 | 0.372** | −0.049 | 0.385** |
| BDE-154 | 0.394** | −0.073 | 0.394** |
| BDE-209 | 0.513** | 0.060 | 0.462** |
| TBB | 0.316** | 0.006 | 0.324** |
| TBPH | 0.335** | 0.025 | 0.341** |
| Organophosphate flame retardants (PFRs) | | | |
| TCEP | 0.375** | −0.013 | 0.343** |
| TDCIPP | 0.386** | −0.099 | 0.397** |
| TCIPP | 0.330** | −0.041 | 0.290** |
| TPHP | 0.247** | −0.011 | 0.199** |

Spearman's correlations between concentrations of select brominated and organophosphate flame retardants (BFRs and PFRs, respectively) and adipogenic activity metrics (triglyceride accumulation per well relative to maximal rosiglitazone response, DNA content/pre-adipocyte proliferation relative to differentiated vehicle control, and triglyceride accumulation per cell normalized to DNA content) as determined using the 3T3-L1 cell culture assay. * = $p < 0.01$, ** = $p < 0.001$, using GraphPad Prism 7.0.

tested between 100 and 1000 μg/mL, and 58% when tested between 1000 and 10,000 μg/mL (Fig. 1B). Again, the efficacies of proliferation also increased with increasing concentration; when diluted extracts were tested at concentrations from 10 to 100 μg/mL, 39% exhibited 7–15% increased DNA content, 13% exhibited 15–30%, and 3% exhibited 30–50%+, compared to when tested at 100–1000 μg/mL, 33% exhibited 7–15% increased DNA content, 23% exhibited 15–30%, and 12% exhibited 30–50% + (Fig. 1B). Triglyceride accumulation and pre-adipocyte proliferation appeared to exhibit differing but overlapping mechanisms (Fig. 2). Certain samples exhibited robust triglyceride accumulation and proliferation (Fig. 2A–C), others exhibited robust triglyceride accumulation and minimal or no proliferation (Fig. 2D–F), and still others exhibited minimal or no triglyceride accumulation but robust proliferation (Fig. 2G–I). Classical peripheral and/or central triglyceride accumulation was observed across active dust extracts as additional visual confirmation of adipogenic effects, while dust controls exhibited no significant activity relative to the differentiated vehicle control (Fig. S2).

### *3.2. Association of adipogenic activity with chemical concentrations in house dust extracts*

Previous work in our laboratory characterized the concentrations of 12 flame retardants in matched dust samples (Hoffman et al., 2017a). These flame retardants (8 BFRs and 4 PFRs) were correlated with extent of maximal adipogenic activity induced by matched dust extracts in 3T3-L1 bioassays. Concentration of the FRs varied, with more than an order of magnitude difference in concentration ranges across chemicals (Table S2). PFRs were generally present at higher concentration than most BFRs, with 5–10-fold higher mean concentrations and up to 30-fold higher median concentrations (Table S2). Statistically significant correlations were reported between each individual FR and both total triglycerides per well and normalized triglycerides per cell (Table 2). None of the BFRs and PFRs were significantly correlated with dust-induced pre-adipocyte proliferation, again suggesting differing but overlapping mechanisms may mediate these two metrics.

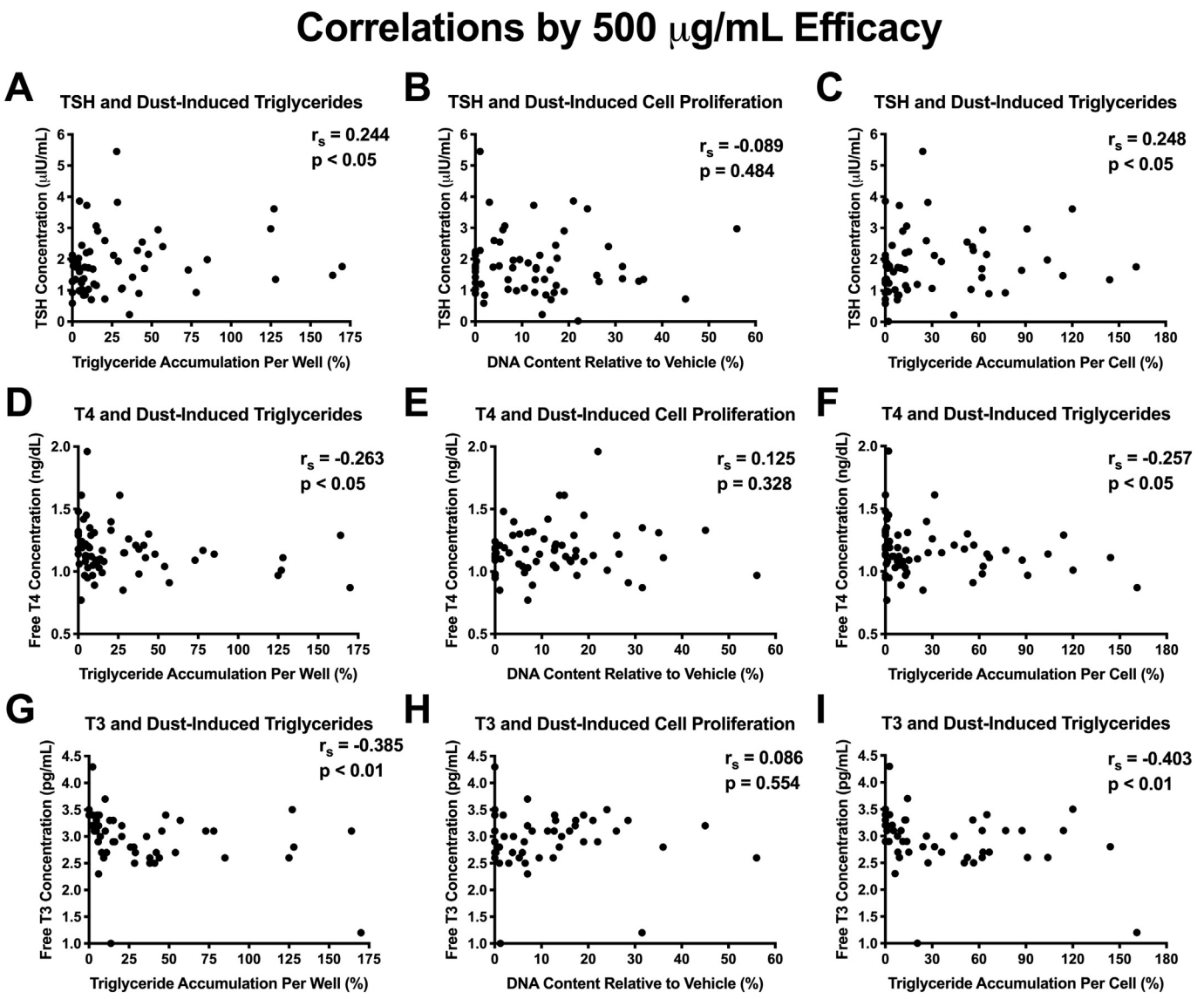


**Fig. 3.** Correlations of adipogenic activity with thyroid hormone status in resident adults. Spearman's correlations comparing serum thyroid hormone measurements in adults with paired dust extract-induced adipogenic activity. (A-C) Serum thyroid stimulating hormone (TSH) concentrations,(D-F) free serum thyroxine (T4) concentrations, (G-I) and free serum triiodothyronine (T3) concentrations. The left column (A, D, G) represents raw triglyceride accumulation per well relative to maximal response for rosiglitazone), the middle column (B, E, H) represents increase (pre-adipocyte proliferation) in DNA content relative to vehicle control, and the right column (C, F, I) represents percent normalized triglyceride accumulation per cell relative to maximal rosiglitazone response (normalized to DNA content). Triglyceride accumulation/cell proliferation values were normalized to responses at 500 μg/mL dust concentration and extrapolated where necessary to correct for varying dust concentrations that did not reach this concentration (n = 28). Significant correlations were determined with $p < 0.05$ using GraphPad Prism 7.0.

### 3.3. Association of adipogenic activity with metabolic health of resident adults

In an attempt to determine whether maximal adipogenic activity was primarily driven by higher dust mass samples (*i.e.* homes where more dust had been able to be collected), exploratory analyses were performed between adipogenic activities and metabolic health metrics, characteristics of which were described in greater detail previously (Hoffman et al., 2017a). Reflecting the original study design, 23% of the participants were male, and participants generally exhibited a median BMI of 25.92 (17.68–49.34), a median age of 48.4 (26-80), a mean educational attainment of 16 years (college grad; 10–23+), and were approximately 78% white, 15% black, and 7% other.

First, correlation analyses were performed between bioactivities and extracted dust masses. Total and normalized triglyceride accumulation were positively correlated with the dust mass concentration ($r_s > 0.45$ and $p < 0.0001$ for each; Table 1, Fig. S3); as a result, normalized metrics were created for further assessment: efficacy of response at 500 μg/mL dust extract concentration and potency (μg/mL extract concentration) at which 10% response was observed (described further in Methods).

Health metrics varied across the cohort (Table S3). Increasing dust extract-induced triglyceride accumulation was correlated with circulating serum thyroid hormones measured in adult residents. Efficacies of triglyceride accumulation (per well and per cell) were positively correlated with serum TSH ($r_s > 0.24$, $p < 0.05$) and negatively correlated with serum FT4 ($r_s > -0.25$, $p < 0.05$) and serum FT3 ($r_s > -0.38$, $p < 0.01$; Fig. 3). Results of regression analyses controlling for participant age, sex, race, and educational attainment suggest similar patterns of association, although associations were not significant in all cases (Table 3). For example, a 10% increase in triglyceride accumulation (per well) was associated with a 0.042 μIU/mL increase in serum TSH of residents (95% confidence interval (CI): −0.06, 0.89; $p < 0.10$). Correlations and regression results were mirrored with triglyceride accumulation potencies (inverse effect, as smaller potency values represent greater potency strength): trends for negative correlations were observed with serum TSH and positive correlations with serum FT4 and FT3 (Table 3, Fig. S4). Pre-adipocyte proliferation efficacies and potencies were not associated (*via* regressions or correlations) with any thyroid hormone measurements, again supporting the putative overlapping but distinct mechanisms mediating these two metrics.

Increasing dust extract-induced triglyceride accumulation was also positively associated with the BMI of residents (each 10% increase in normalized triglyceride accumulation was associated with a 0.29 kg/m$^2$ increase in BMI (CI 0.11, 0.48; $p < 0.01$; Fig. 4, Table 3). Potencies of triglyceride accumulation were not associated with BMI, nor were triglyceride accumulation efficacies or potencies correlated with BMI (Spearman's correlations; Figs. S5, S6). Pre-adipocyte proliferation efficacies tended to be positively correlated with BMI ($r_s > 0.16$, $p < 0.10$; Fig. S5) and negatively correlated with pre-adipocyte proliferation potencies ($r_s > 0.17$, $p < 0.10$; Fig. S6), although adjusted regressions did not support this association (Table 3).

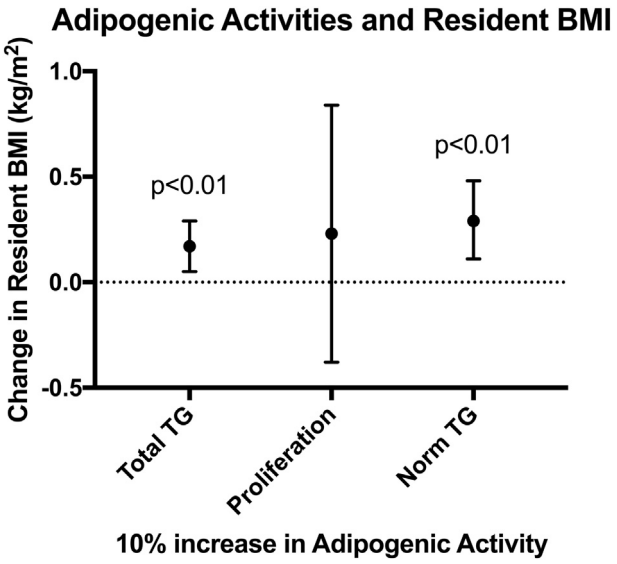


**Fig. 4.** Dust-induced triglycerides associated with BMI of adult residents. Robust regressions were performed to assess relationships between adipogenic activity metrics at 500 μg/mL dust extract concentrations (total triglyceride accumulation per well, pre-adipocyte proliferation, and normalized triglyceride accumulation per cell) and resident body mass index (BMI). These models controlled for potential confounding by age, sex, race, and education, which we anticipated could be related to health outcomes as well as the types of contaminants in the home environment driving the measured adipogenic activity. Analyses were performed using SAS 9.4. Adipogenic metrics were scaled such that a 10% increase in triglyceride accumulation (relative to positive control) or pre-adipocyte proliferation (relative to differentiated vehicle control) was associated with the change in BMI depicted in the figure.

**Table 3**
Regressions of resident health outcomes and adipogenic activities.

| Dependent variable | Adipogenic metric | Measure | Estimate | 95% Confidence limits | p Value |
|---|---|---|---|---|---|
| BMI | Total Triglycerides | Efficacy[a] | 0.17 | 0.05, 0.29 | <0.01 |
| | | *Potency*[b] | *−0.01* | *−0.05, 0.02* | *0.37* |
| | Pre-adipocyte Proliferation | *Efficacy*[a] | *0.23* | *−0.38, 0.84* | *0.47* |
| | | *Potency*[b] | *−0.02* | *−0.27, 0.24* | *0.91* |
| | Normalized Triglycerides | Efficacy[a] | 0.29 | 0.11, 0.48 | <0.01 |
| | | *Potency*[b] | *−0.01* | *−0.06, 0.04* | *0.71* |
| TSH | Total Triglycerides | Efficacy[a] | 0.042 | −0.01, 0.09 | 0.08 |
| | | *Potency*[b] | *−0.01* | *−0.03, 0.01* | *0.39* |
| | Pre-adipocyte Proliferation | *Efficacy*[a] | *0.12* | *−0.05, 0.30* | *0.17* |
| | | *Potency*[b] | *0.01* | *−0.03, 0.06* | *0.60* |
| | Normalized Triglycerides | *Efficacy*[a] | *0.04* | *−0.01, 0.09* | *0.11* |
| | | *Potency*[b] | *−0.01* | *−0.02, 0.01* | *0.33* |
| FT4 | Total Triglycerides | *Efficacy*[a] | *−0.01* | *−0.02, 0.003* | *0.14* |
| | | Potency[b] | 0.01 | 0.002, 0.01 | <0.01 |
| | Pre-adipocyte Proliferation | *Efficacy*[a] | *0.01* | *−0.04, 0.05* | *0.80* |
| | | *Potency*[b] | *−0.01* | *−0.02, 0.003* | *0.22* |
| | Normalized Triglycerides | *Efficacy*[a] | *−0.01* | *−0.02, 0.003* | *0.14* |
| | | *Potency*[b] | *−0.002* | *−0.004, 0.001* | *0.14* |
| FT3 | Total Triglycerides | *Efficacy*[a] | *−0.01* | *−0.04, 0.02* | *0.48* |
| | | *Potency*[b] | *0.01* | *−0.003, 0.02* | *0.13* |
| | Pre-adipocyte Proliferation | *Efficacy*[a] | *−0.003* | *−0.09, 0.09* | *0.96* |
| | | *Potency*[b] | *−0.002* | *−0.02, 0.01* | *0.83* |
| | Normalized Triglycerides | *Efficacy*[a] | *−0.02* | *−0.04, 0.01* | *0.27* |
| | | *Potency*[b] | *0.004* | *−0.002, 0.01* | *0.20* |

Robust regressions (as described in (Hampel et al., 1986; Yohai, 1987)) were performed to assess relationships between adipogenic activity metrics (as determined by the 3T3-L1 cell culture assay; triglyceride accumulation per well relative to maximal rosiglitazone response, DNA content/pre-adipocyte proliferation relative to differentiated vehicle control, and triglyceride accumulation per cell normalized to DNA content) and resident health outcomes (body mass index (BMI), thyroid stimulating hormone, free triiodothyronine (T3), and free thyroxine (T4)). These models included age, sex, race, and education as potential confounding variables. Analyses were performed using SAS 9.4. N = 137 for BMI and 69 for thyroid hormone measurements.
Italicized values denote non-significant regressions.
[a] Efficacy estimates and confidence intervals provided as per 10% change in triglyceride accumulation or pre-adipocyte proliferation percent.
[b] Potency estimates and confidence intervals provided as per 100 μg/mL change in concentration at which triglyceride accumulation or pre-adipocyte proliferation reached 10% effect.

### 3.4. Role of TRβ antagonism in adipogenic activity of house dust extracts

Based on adipogenic activity associations with thyroid hormone levels, TRβ antagonism was evaluated as a potential contributory factor in the observed adipogenic activities; TR has been demonstrated to be expressed in these cells by several researchers previously, and increases throughout differentiation (Fu et al., 2005; Jiang et al., 2004). The TR antagonist, 1–850, stimulates a robust triglyceride accumulation response relative to rosiglitazone, a PPARγ agonist (Fig. 5A)

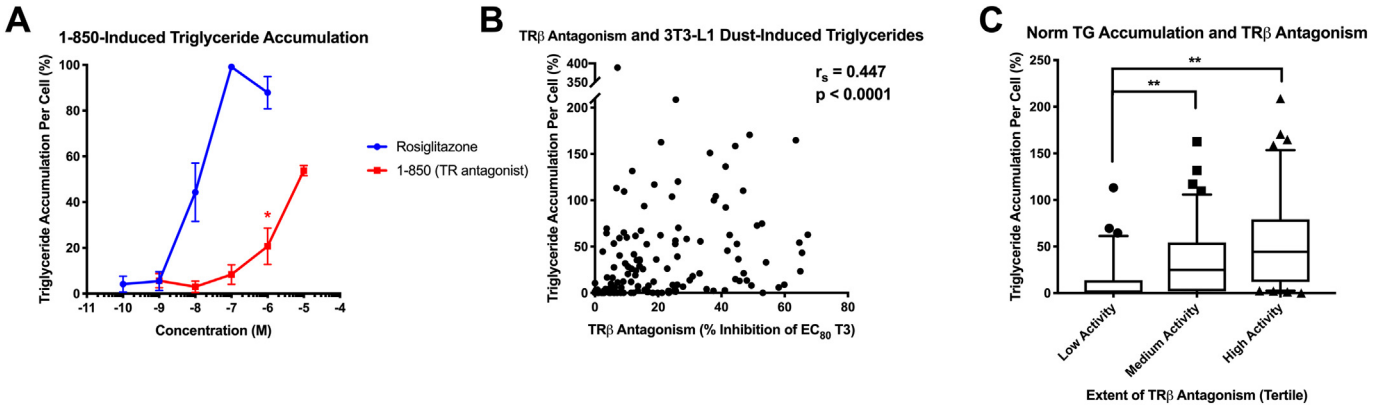


**Fig. 5.** Putative role of thyroid receptor beta antagonism in dust extract-induced adipogenic activity. Putative interrogation of thyroid receptor beta (TRβ) antagonism as a molecular mechanism promoting triglyceride accumulation. (A) Normalized triglyceride accumulation per cell relative to maximal intra-assay response for rosiglitazone (normalized to DNA content) for 1–850, a TR antagonist. 3T3-L1 cells were induced to differentiate as described in Methods and assessed for degree of adipocyte differentiation after ten days of differentiation. * denotes the lowest concentration at which a significant increase above baseline values (differentiated DMSO control) was observed. (B) Spearman's correlation comparing normalized triglyceride accumulation per cell relative to maximal intra-assay response for rosiglitazone (normalized to DNA content) versus TRβ antagonism as measured via GeneBLAzer TRβ FRET reporter assay (Invitrogen) (Kollitz et al. 2018). Significant correlations were determined with $p < 0.05$ using GraphPad Prism 7.0. (C) Box and whisker plots depicting normalized triglyceride accumulation per cell (normalized to DNA content) in each tertile of maximal TRβ antagonism (low/medium/high antagonist activities). **denotes significant differences between groups as per Kruskal-Wallis test, $p < 0.001$, using GraphPad Prism 7.0.

(Kassotis et al., 2017a). Concurrent research in our laboratory evaluated the thyroid receptor agonism/antagonism by these same dust extracts using a cell-based reporter assay system (Kollitz et al., 2018) and the extent of TRβ antagonism was positively correlated with normalized triglyceride accumulation ($r_s = 0.44$, $p < 0.0001$), supporting a potential contributory role of TRβ in adipogenesis (Fig. 5B). TR antagonism was not correlated with pre-adipocyte proliferation, further delineating the mechanisms mediating these adipogenic outcomes. Two targeted experiments were thus carried out to attempt to confirm this contributory mechanism to mixture-induced triglyceride accumulation. Nine dust extracts exhibiting moderate triglyceride accumulation and TRβ antagonism were selected for targeted mechanistic testing. First, these extracts were tested at low and high concentrations either alone or co-exposed with 10 μM T3; we hypothesized that if TRβ antagonism was promoting triglyceride accumulation, addition of a TR agonist would counteract this response. At low-dose extract concentrations (Fig. 6A, C, E), T3 co-exposure significantly reduced dust-induced triglyceride accumulation per cell in six of nine samples ($p < 0.05$) and tended to inhibit in one more ($p < 0.10$; Fig. 6A, E). One sample appeared to have no effect on triglyceride accumulation, and one sample increased accumulation rather than decreased it. Three samples exhibited significant decreases in pre-adipocyte proliferation following T3 co-exposure ($p < 0.05$), while no effects were observed for the other six samples (Fig. 6C). Results at high dust extract concentrations (Fig. 6B, D, F) were less marked than those at lower concentrations, with only three samples exhibiting a significant decrease in triglyceride accumulation per cell ($p < 0.05$) and tended to inhibit in one more ($p < 0.10$; Fig. 6B, F). One sample exhibited increased triglyceride accumulation per cell with T3 co-exposure, and two samples exhibited reduced pre-adipocyte proliferation (Fig. 6D).

A confirmatory mechanistic test assessed this relationship *via* siRNA knock-down of TRα/TRβ.; we hypothesized that a decrease in available receptors (using a knock-down approach) would also reduce TR antagonism-driven triglyceride accumulation. The same nine dust extracts were assessed at the low concentration, using: dust alone, dust in cells treated with a scrambled siRNA negative control, and dust in cells treated with a TRα/TRβ siRNA knock-down protocol. To rule out potential inhibitory effects of TR knock-down on adipogenesis itself, vehicle controls and rosiglitazone responses were compared across siRNA treatments: no differences were observed between non-transfected controls and siRNA treatments (Fig. S7A). To functionally determine the effect of knocking down TR before assessing the effects on dust, we examined full dose responses of 1–850 (a potent and selective TR antagonist) across these treatments. Importantly, while no differences were observed in 1–850-induced triglyceride accumulation between the naïve and scrambled siRNA cells, the TR-siRNA cells exhibited approximately a 67% reduction in triglyceride accumulation at the highest concentration of 1–850 tested ($p < 0.05$; Fig. S7B–D), similar to the magnitude of effect observed previously using this protocol to knock-down PPARγ (Foley et al., 2015). Following confirmation of vehicle and control chemical responses, we further assessed putative effects by the transfection process on dust-induced responses. We observed no significant differences between the control (dust alone) and the scrambled siRNA negative control for any dust extract (Fig. S8). Finally, the TR knock-down significantly reduced dust-induced triglyceride accumulation per cell in five of nine extracts ($p < 0.05$), tended to reduce in three more ($p < 0.10$), and resulted in a non-significant reduction in the final extract (Fig. 7A, C). No significant impacts on DNA content were observed, though one extract tended to increase DNA content following siRNA knock-down ($p < 0.10$; Fig. 7B).

## 4. Discussion

These results suggest that complex mixtures in house dust induce adipogenic activity *in vitro*, at environmentally relevant concentrations, and that this activation is controlled, at least in part, by antagonism of TRβ. Furthermore, the adipogenic activity observed in a large number of indoor house dust sample extracts was associated with the BMI and serum thyroid hormone levels of adult residents living in these homes. 90% of the tested dust extracts exhibited significant adipogenic activity *via* either increased triglyceride accumulation and/or pre-adipocyte proliferation. Importantly, >40% of samples exhibited significant effects at concentrations <10 μg dust/well, suggesting a potent biological response by the mixtures of contaminants present in dust, particularly notable given that the EPA estimates that children ingest 60–100 mg of dust per day from indoor environments (U.S. Environmental Protection Agency (EPA), 2017). While translation of oral human exposure to *in vitro* effects is not clear, the wide disparity in concentrations warrants further research to investigate potential *in vivo* effects.

The extent of triglyceride accumulation was highly positively correlated with the extracted dust masses, which is likely due to the inherently greater chemical contaminant concentrations in each assay well associated with this, and likely the causative factor in these observed effects. Interestingly, the extent of pre-adipocyte proliferation was not

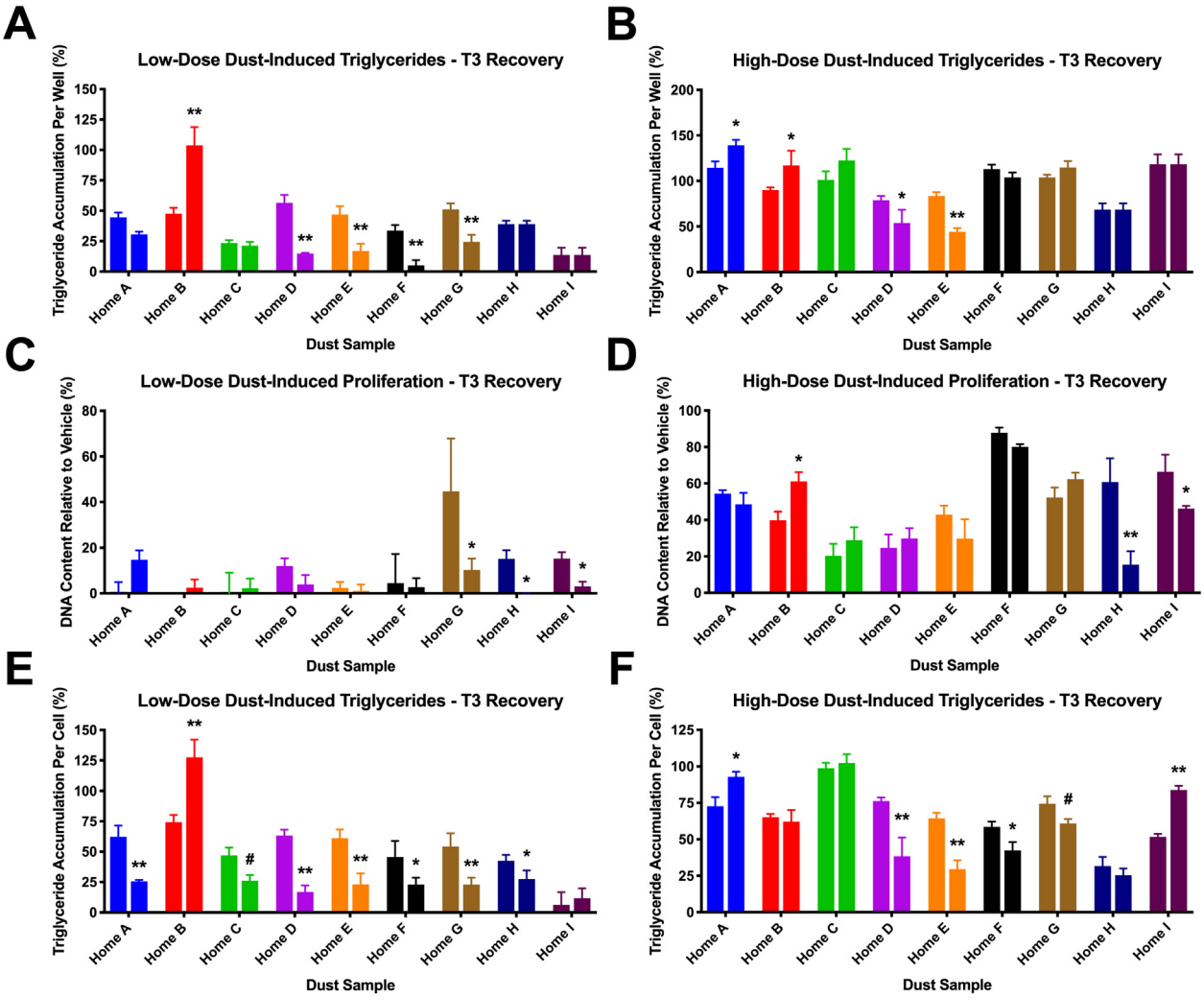


**Fig. 6.** Co-exposure to T3 inhibits dust-induced triglyceride accumulation. 3T3-L1 cells were induced to differentiate as described in Methods. Cells were assessed for degree of adipocyte differentiation after ten days of differentiation while exposed to set concentrations of nine select indoor house dust extracts either alone or co-treated with 10 μM T3. (A, B) Raw triglyceride accumulation per well relative to maximal response for rosiglitazone, (C, D) increase (pre-adipocyte proliferation) in DNA content relative to vehicle control, and (E, F) percent normalized triglyceride accumulation per cell relative to maximal rosiglitazone response (normalized to DNA content). Two concentrations were examined for each dust extract. The left column depicts low-dose concentrations that were 16-fold dilutions of the maximal concentrations for each extract (A, C, E). The right column depicts high-dose concentrations tested were the maximal concentration of each extract and varied depending on the quantity of dust collected in each household (B, D, F). The left bar in each pair depicts the response induced by the dust extract alone, while the right bar in each pair depicts the activity induced by the dust extract co-treated with T3. Data presented as mean ± SEM from two independent experiments of four biological replicates. # = $p < 0.10$, * = $p < 0.05$, ** = $p < 0.01$ indicate statistical differences between T3/dust co-treatment and dust samples alone as per one-way ANOVA and Dunnett's posthoc test in GraphPad Prism 7.0.

correlated with extracted dust masses; this may be due to differing assay sensitivities (lower dynamic range for proliferation *vs.* triglyceride accumulation, for example). Despite this, pre-adipocyte proliferation appeared to be a more sensitive metric than triglyceride accumulation (though notably these endpoints measure distinct but related processes), with a greater percentage of samples between 1 and 10 and 10–100 μg/mL exhibiting significant pre-adipocyte proliferation than triglyceride accumulation. Further research is needed to more comprehensively evaluate the apparent overlapping but distinct molecular mechanisms driving these two effects. Neither adipogenic metric was significantly different between dust samples from thyroid cancer cases relative to dust samples from control homes (the source of the dust in this study; (Hoffman et al., 2017a)).

A notable finding of this study was that the extent of triglyceride accumulation was positively correlated with the concentrations of each of twelve BFRs and PFRs measured in the dust. Despite this, the majority of these contaminants were previously demonstrated to be inactive individually in this same cell system in our lab (Kassotis et al., 2017b); 2,2′,4,4′,-tetrabromodiphenyl ether (BDE-47), triphenyl phosphate (TPHP), and bis (2-ethyl hexyl)-2,3,4,5-tetrabromophthalate (TBPH) demonstrated weak to moderate triglyceride accumulation and/or pre-adipocyte proliferation. However, these chemicals only account for a small fraction of the observed activity given the concentrations of these contaminants in the dust extracts. This, coupled with the significant correlations with other inactive chemicals, suggests that flame retardants are not the primary drivers of the observed activity. We hypothesize that either mixture effects or other co-occurring contaminants are primarily responsible for the effects reported herein. Numerous studies have demonstrated the "something from nothing" and "a lot from a little" effects both *in vitro* (Rajapakse et al., 2002; Silva et al., 2002) and *in vivo* (Runnalls et al., 2015; Thrupp et al., 2018), demonstrating the potential additive or greater than additive effects of complex mixtures. Another potential concern is that the presence of multiple chemicals in a mixture likely increases the chance of inducing the expression of cytochrome P450s and other bioactivation enzymes (Vanella et al., 2011; De Taeye et al., 2010; Guo and Liao, 2000). For example, previous work demonstrated that several PCB congeners were inactive thyroid receptor ligands when dosed individually; however,

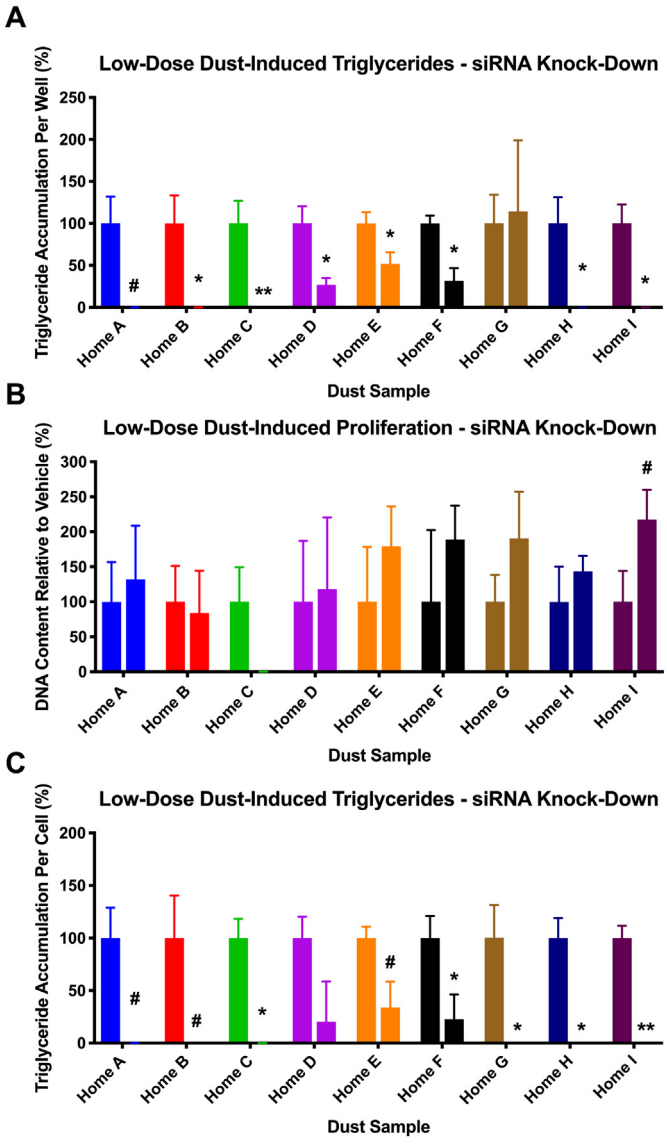


**Fig. 7.** siRNA knock-down of thyroid receptor expression inhibits dust-induced triglyceride accumulation. 3T3-L1 cells were transfected with either no siRNA, a negative control (scrambled) siRNA, or TRα and TRβ siRNAs and were then induced to differentiate as described in Methods. Cells were assessed after ten days of differentiation while exposed to set concentrations (16-fold dilutions of maximal concentrations) of nine select indoor house dust extracts. (A) Raw triglyceride accumulation per well relative to maximal response for rosiglitazone, (B) increase in pre-adipocyte proliferation (DNA content) relative to vehicle control, and (C) percent normalized triglyceride accumulation per cell relative to maximal rosiglitazone response (normalized to DNA content). The left bar in each pair depicts the response induced by the dust extract alone, while the right bar in each pair depicts the activity induced by the dust extract following transfection with TRα and TRβ siRNAs. Data presented as mean ± SEM from two independent experiments of four biological replicates. # = $p < 0.10$, * = $p < 0.05$, ** = $p < 0.01$ indicate statistical differences between TR siRNA and dust control samples as per one-way ANOVA and Dunnett's posthoc test in GraphPad Prism 7.0. No significant differences were observed between the dust control samples and the negative control (scrambled) siRNA; see Supplemental Figs. 7 and 8 for additional details on these results.

they were metabolized into bioactive compounds that promoted TR-mediated effects when present in mixtures that induced expression of CYP1A1, using a rodent pituitary cell line (Gauger et al., 2007; Giera et al., 2011). House dust contains thousands of chemicals (Ferguson et al., 2015; Hilton et al., 2010), so it is likely that there are other active constituents that have yet to be identified and measured. For example, our lab published previously on other common house dust contaminants, reporting much greater adipogenic activity for other contaminants not measured herein (Kassotis et al., 2017b; Ferguson et al., 2015; Kassotis et al., 2018). More research is needed to further investigate the primary chemicals or effects driving this. Other chemicals as well as metals are also known to accumulate in house dust (Rasmussen et al., 2013), and have also been demonstrated to modulate adipogenesis/adiposity (Lee et al., 2012; Martini et al., 2014; Park et al., 2017), suggesting a potential multifactorial molecular mechanism. The fact that none of the quantified/identified/measured BFRs or PFRs were significantly correlated with dust-induced pre-adipocyte proliferation supports our hypothesis that overlapping but distinct mechanisms are promoting these two adipogenic metrics.

This study is the first to report an association between dust-induced triglyceride accumulation *in vitro* and both BMI and thyroid hormone concentrations of adult residents living in sampled homes. Triglyceride accumulation per well and per cell were positively associated with BMI using a robust regression model ($\beta = 0.29$ per 10% change in triglyceride accumulation, 95% CI 0.11–0.48, $p < 0.01$). While these results suggest that the extent of adipogenic chemicals present in the dust are associated with greater weights of residents living in those homes, these analyses are exploratory and more research with a greater sample size is needed. This association could be due to chronic exposure to chemicals in the indoor environment acting directly on the residents to alter: hormonal control of appetite and/or satiety, basal metabolic rate, adipocyte commitment and/or differentiation, the brain circuitry that controls food intake and/or energy expenditure, or by shifting energy balance to favor caloric storage, as reviewed previously (Heindel et al., 2017). Future research is needed to more definitively examine this association, examine this potential association in children who have a more permanent link to their indoor environment and are in the process of developing their metabolic regulatory systems, better assess causative molecular mechanisms for the pre-adipocyte proliferation metric, and identify putative causative chemicals. Dust-induced triglyceride accumulation efficacies also appeared to be positively correlated with serum TSH (suggesting increased dust-induced adipogenic activity *in vitro* was associated with higher TSH), and negatively correlated with serum FT3 and FT4, although regression results adjusting for additional covariates were not statistically significant. However, both analyses (*i.e.*, correlation and regression) showed the same direction of effect, bolstering a potential association, although our sample size may have limited our ability to detect statistically significant associations in the adjusted models. Notably, our previous work found that approximately half of these same extracts antagonized T3-mediated TRβ activity by 20–67% and that TRβ antagonist potencies were negatively correlated with serum FT4 ($p < 0.001$) and serum FT3 ($p < 0.10$) concentrations in residents, suggesting a TR-mediated impact on thyroid function (Kollitz et al., 2018). In addition, we've previously demonstrated that treatment of 3T3-L1 cells with the TR antagonist 1–850 induced a robust triglyceride accumulation response (Kassotis et al., 2017a), providing support for this as a causative mechanism herein, supporting the associations with thyroid hormone measurements, and providing a plausible mechanism through which thyroid receptor antagonists in the dust may influence both thyroid hormone measurements and BMI of residents and the dust-induced triglyceride accumulation *in vitro*. As before, the degree of dust-induced pre-adipocyte proliferation was not significantly correlated with any thyroid measurements, suggesting that this may not be as important a mechanism for proliferation.

Given a significant positive correlation between the extent of triglyceride accumulation and the extent of TRβ antagonism (Kollitz et al., 2018), two additional targeted experiments were performed to evaluate the role of TR antagonism in the adipogenic effects: a ligand recovery experiment (co-treating cells with dust extracts and T3 to attempt to counteract TR antagonist-induced triglyceride accumulation) and an siRNA knock-down of TR. In this select set of dust extracts, both T3 and siRNA knock-down of TR isoforms succeeded in counteracting a significant amount of the dust-induced triglyceride accumulation for the majority of samples. This suggests TR antagonist binding and

accompanying co-regulator recruitment may mediate these effects, given that TR knock-down did not appear to affect basal differentiation extent nor PPARγ-mediated ligand responses (rosiglitazone), yet did inhibit TR antagonist (1–850)-mediated triglyceride accumulation. These mechanistic data demonstrate TR antagonism as a contributory factor in at least some of the observed adipogenic effects *in vitro* as well as a likely factor in the modulated thyroid hormone concentrations in residents. Lesser effects were observed in the ligand recovery experiment with higher dust extract concentrations, suggesting a greater TR antagonism effect that would have required greater agonist addition to counteract. Further substantiation of this putative mechanism is also needed in a larger collection of dust extracts with varying degrees of TR bioactivities, though it is beyond the scope of the work reported herein. That all said, the TR antagonism does not explain the entirety of the adipogenic activity induced by these dust extracts, which is expected given the complexity of chemicals present in samples of this type. Samples are also heterogeneous (Mitro et al., 2016), with different homes having varying concentrations of specific contaminants, likely due to differing consumer products and construction materials in the home (Hoffman et al., 2015b; Hoffman et al., 2014). More work is needed to better characterize the chemical complexity of indoor dust samples, using both targeted and non-targeted analysis techniques to bolster our understanding of the chemicals and mixtures present in environmental matrices that may contribute to metabolic health dysfunction. Given a wider targeted chemical assessment, future research should utilize a weighted potency approach to assessing mixture effects using more sophisticated analyses. Further work should also examine the precise mechanisms of lipid accumulation by assessing gene expression for various TR endpoints, lipogenesis, lipolysis, *etc.*

In conclusion, we report widespread extract-induced adipogenic activity *in vitro* by a large collection of indoor house dust extracts collected in central NC and tested at environmentally relevant levels. We have further established a likely contributory causative molecular mechanism for the observed effects through TR antagonism, although further research is needed to establish the relative contribution of this mechanism as well as to examine this relationship *in vivo*. Importantly, this work establishes an important role for TR antagonism in complex environmental mixtures modulating metabolic dysfunction *in vitro*, a mechanism that has received limited attention previously. Further, *in vitro* triglyceride accumulation was positively associated with BMI and serum TSH and tended to be negatively associated with serum FT3 and FT4 levels in residents, raising concerns that these complex mixtures of indoor contaminants could affect metabolic health (*via* modulation of key metabolic hormones and/or adiposity). Notably, thyroid health analyses were performed only in individuals with no history of thyroid dysfunction; as such, it is possible we have removed those individuals most sensitive to environmental exposures, limiting our ability to detect potential impacts. Our study population included a relatively small sample size and a somewhat homogenous study population (reported previously in detail (Hoffman et al., 2017a)), which might therefore limit the generalizability to the overall population; concentration of various contaminants in dust are also expected to vary geographically and temporally (Hoffman et al., 2017b; Hoffman et al., 2017c), so future research should attempt to reproduce these effects utilizing dust from other regions and settings. We are also limited by the cross-sectional study design, wherein we measured contaminants in the home at the same time we measured BMI, thyroid hormones, and other health metrics; however, we restricted participation to residents living in the same home for at least two years to ensure exposure measurements captured a longer-term environmental exposure (residents in our study lived in homes on average eleven years) (Hoffman et al., 2017a). These results highlight a need for additional studies to examine further how exposure to dust extracts, and particularly exposure to mixtures of household contaminants, affects adipogenesis *in vitro*, *in vivo,* and subsequently, could affect the health of the general population.

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## Competing interests declaration

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.02.273.

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