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An adipose IncRAP2-Igf2bp2 complex enhances adipogenesis and energy expenditure by stabilizing target mRNAs

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Highlights

IncRAP2 is a cytosolic IncRNA conserved in mouse and human needed for adipogenesis

IncRAP2 complexes with mRNA stability and translation regulators, including Igf2bp2

IncRAP2-Igf2bp2 stabilizes lipid metabolism mRNAs to potentiate energy expenditure

IncRAP2-Igf2bp2 genetic and expression variation is linked to BMI, type 2 diabetes
An adipose IncRAP2-Igf2bp2 complex enhances adipogenesis and energy expenditure by stabilizing target mRNAs

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SUMMARY
IncRAP2 is a conserved cytoplasmic lncRNA enriched in adipose tissue and required for adipogenesis. Using purification and in vivo interactome analyses, we show that IncRAP2 forms complexes with proteins that stabilize mRNAs and modulate translation, among them Igf2bp2. Surveying transcriptome-wide Igf2bp2 client mRNAs in white adipocytes reveals selective binding to mRNAs encoding adipogenic regulators and energy expenditure effectors, including adiponectin. These same target proteins are downregulated when either Igf2bp2 or IncRAP2 is downregulated, hindering adipocyte lipolysis. Proteomics and ribosome profiling show this occurs predominantly through mRNA accumulation, as IncRAP2-Igf2bp2 complex binding does not impact translation efficiency. Phenome-wide association studies reveal specific associations of genetic variants within both IncRAP2 and Igf2bp2 with body mass and type 2 diabetes, and both IncRAP2 and Igf2bp2 are suppressed in adipose depots of obese and diabetic individuals. Thus, the IncRAP2-Igf2bp2 complex potentiates adipose development and energy expenditure and is associated with susceptibility to obesity-linked diabetes.

INTRODUCTION
Pervasive transcription of the human and mouse genomes generates thousands of long noncoding RNAs (lncRNAs), but only a small minority have been linked to specific biochemical functions. Previous studies revealed that many lncRNAs are specifically enriched in white and/or brown adipocytes and play vital roles in adipocyte biology (Knoll et al., 2015; Sun and Lin, 2019), including in adipogenesis, thermogenesis, and insulin sensitivity. As example, we and others showed that the brown fat-specific lncRNAs Blnc1 (Zhao et al., 2014) and lncBATE1 (Alvarez-Dominguez et al., 2015) interact with the nuclear matrix factor hnRNPU to mediate trans-activation of genes mediating brown and/or beige thermogenic programs.

While lncRNAs can potentially bind DNA, RNA, or protein targets, much work suggests that lncRNA mechanisms predominantly involve binding to proteins, either as scaffolds for ribonucleoprotein complexes or as decoys that prevent their assembly (Alvarez-Dominguez and Lodish, 2017; Wang and Chang, 2011). To better understand how lncRNAs function, several technologies have been recently developed for unbiased determination of lncRNA localization, protein targets, and functional domains (Goff and Rinn, 2015; McDonnell and Guttman, 2019). Single-molecule fluorescence in situ hybridization (smFISH) visually determines RNA abundance and location, revealing whether a lncRNA diffuses to trans sites beyond its chromosomal locus or remains tethered in cis in the nucleus (Cabili et al., 2015; Raj et al., 2008). Cross-linking intact cells followed by hybridization-based RNA purification captures the specific DNA, RNA, or protein targets to which a lncRNA binds in vivo (rather than in solution after cell lysis) (Chu et al., 2011, 2015; Engreitz et al., 2013; Simon et al., 2013). And footprint profiling can map the RNA sequence sites where proteins bind lncRNAs, revealing their functional domains (Darnell, 2010; Ingolia et al., 2009; Silverman et al., 2014).

Here, we use these and other tools to interrogate the mode of action of IncRAP2, a white adipocyte-selective RNA that is essential for adipogenesis (Sun et al., 2013). We show that IncRAP2 predominantly resides in the cytoplasm, yet it does not directly associate with ribosomes or other RNAs. Instead, IncRAP2 forms a complex with several RNA-binding proteins that affect mRNA stability and translation. Among these is Igf2bp2, which has been implicated in posttranscriptional control of metabolically important proteins.
Figure 1. IncRAP2 is a conserved cytoplasmic RNA required for adipogenesis

(A and B) IncRAP2 is a capped, polyadenylated, and spliced RNA transcribed from a promoter bound by PPARγ and C/EBPa. Tracks show signal from sequencing studies of white adipocytes from mouse (A) and human (B) (Data S4).

(C and D) IncRAP2 is adipocyte-specific and strongly induced during adipogenesis. Shown are relative tissue expression (normalized to spleen, left) and relative induction during in vitro differentiation of white (center) or brown (right) preadipocytes from mouse (C) and human (Ding et al., 2018) (D). scWAT, subcutaneous adipose tissue; eWAT, epididymal white adipose tissue; BAT, brown adipose tissue.

(E and F) IncRAP2 depletion blocks adipogenesis. Shown are lipid accumulation by Oil-red O staining (E) and relative expression of key adipocyte genes (F) in day 6 differentiated white adipocytes pretreated with control or two different IncRAP2-targeting DsiRNAs.

(F) Induction during in vitro differentiation of brown adipocytes shown are relative tissue expression (normalized to spleen, left) and relative induction during in vitro differentiation of white (center) or brown (right) preadipocytes from mouse (C) and human (Ding et al., 2018) (D). scWAT, subcutaneous adipose tissue; eWAT, epididymal white adipose tissue; BAT, brown adipose tissue.

(E and F) IncRAP2 depletion blocks adipogenesis. Shown are lipid accumulation by Oil-red O staining (E) and relative expression of key adipocyte genes (F) in day 6 differentiated white adipocytes pretreated with control or two different IncRAP2-targeting DsiRNAs.
Figure 1. Continued
(G and H) IncRAP2 localizes to the cytoplasm. Relative expression in fractionated nuclear and cytoplasmic compartments (G) and single-molecule FISH detection (H) in day 6 differentiated white adipocytes. IncRAP2 molecules above nucleus overlay DAPI staining in maximum z stack projections of FISH images, quantified to the right (n = 9 cells with ≥ 1 transcripts). **p <0.01, ***p <0.001 (t-test).

(Dai et al., 2015; Laggai et al., 2014; Zhang et al., 2018). We identify the transcriptome-wide Igf2bp2 mRNA clients in white adipocytes, which include key adipogenic effectors and mediators of energy metabolism. Indeed, we show that depleting either IncRAP2 or Igf2bp similarly downregulates the proteins encoded by these targets, including adiponectin, and that this occurs primarily through mRNA destabilization. Accordingly, adipocytes in which either IncRAP2 or Igf2bp is depleted show compromised energy expenditure. We further find that the levels of IncRAP2 and Igf2bp2 in adipocytes are reduced during the development of obesity and diabetes. Analysis of genome-wide association studies reveals a specific association of IncRAP2 and Igf2bp2 polymorphic alleles with increased body fat and greater risk of type 2 diabetes. Thus, a previously uncharacterized IncRAP2-Igf2bp2 complex regulates adipose energy expenditure, with implications for the susceptibility to and pathogenesis of obesity-linked diabetes in humans.

**RESULTS**

**IncRAP2 is a conserved, adipose-enriched cytoplasmic lncRNA required for adipogenesis**

We previously identified several lncRNAs common to white and brown adipose that, based on effects of depleting them in mouse preadipocytes, are essential for adipocyte development and function (Sun et al., 2013). One of them, IncRAP2 (GenBank: NR_040299.1), is transcribed from an intergenic promoter bound by PPARγ, C/EBPα, and C/EBPβ (Figures 1A and S1A). 5'/3'0 RACE verifies a capped and polyadenylated, 6.8kb spliced RNA (Figure S1B). IncRAP2 is highly adipose tissue-specific and strongly induced during early white and brown adipogenesis (Figure 1C). Notably, IncRAP2's structure, regulation, and expression traits are conserved in humans (Figures 1B and 1D). Depleting IncRAP2 by ~70%–75% by transducing Dicer-substrate siRNAs (DsiRNAs) into primary white preadipocytes (Isidor et al., 2016) dramatically blocks their subsequent differentiation in culture, as evidenced by impaired lipid accumulation and blunted induction of key adipocyte genes (PPARγ, C/EBPα, Adiponectin, Fabp4, and Glut4) (Figures 1E and 1F). Single-molecule fluorescence in situ hybridization (smFISH) reveals that IncRAP2 diffuses from the nucleus, spreading throughout the cytoplasm at 14 ± 3 transcripts per white and 9 ± 3 transcripts per brown adipocyte (Figure 1H). Cell fractionation verifies its cytoplasmic localization, unlike IncRAP1 (also called FIRRE) (Figure 1G), which mediates nuclear trans-chromosomal interactions among adipogenic genes (Hacisuleyman et al., 2014). These results show that IncRAP2 is a conserved cytoplasmic RNA essential for adipogenesis.

**IncRAP2 forms complexes with proteins that regulate mRNA stability and translation, including Igf2bp2**

To investigate how IncRAP2 functions, we sought to identify its binding partners and targets. IncRAP2's mRNA-like features suggest that it could bind ribosomes, which we tested by examining parallel RNA and ribosome footprint profiling during mouse adipogenesis (Reid et al., 2017). Despite strong induction during adipogenesis, IncRAP2 is largely devoid of ribosome-protected RNA fragments, and exhibits poor translatability (Figures 2A and 2B). Supporting the notion that IncRAP2 is not translated, no peptides could be found in proteome surveys of brown or white adipocytes from either mouse or human (Alvarez-Dominguez et al., 2015; Desiere et al., 2006). We conclude that IncRAP2 is unlikely to engage translating ribosomes.

To identify IncRAP2's binding targets, we used biotin-labeled smFISH probes to purify IncRAP2 from cross-linked intact mouse 3T3-L1 adipocytes, which express IncRAP2 at levels comparable to those of primary adipocytes (Figure S2A). Using exon-targeting probes, we retrieved ~90% of cellular IncRAP2, whereas less than 1% was retrieved following RNase A treatment, without targeting probes, or with probes targeting introns or unrelated lncRNAs like Bloodlinc (an erythrocyte-specific lncRNA) (Alvarez-Dominguez et al., 2014) or H19 (a broadly expressed lncRNA) (Pachnis et al., 1984) (Figure 2C). These controls attest to the efficiency and specificity of our RNA antisense purification protocol. We then used RNA interactome analysis by sequencing (RIA-seq) (Kretz et al., 2013) to probe interactions of IncRAP2 with other RNAs via glutaraldehyde cross-linking. IncRAP2 was robustly retrieved by distinct pools of exon-targeting probes, but no other RNAs were cross-linked with significant enrichment or with concordance between probe pools (Figures S2B and S2C), indicating that IncRAP2 does not directly bind other RNAs.
We then conducted a comprehensive identification of RNA-binding proteins by mass spectrometry (ChIRP-MS) (Chu et al., 2015) in 3T3-L1 adipocytes using formaldehyde cross-linking, which preserves both direct and indirect RNA-protein interactions. As controls, we compared cross-linked proteins to those captured using RNase A treatment, intron-targeting probes, non-targeting probes, or no probes.

Figure 2. lncRAP2 forms a complex with mRNA stability and translation regulators

(A and B) lncRAP2 does not engage translating ribosomes. Tracks show signal from ribosome profiling, RNA, and RNA interactome sequencing studies of differentiated white adipocytes (A). Data are pooled from n = 2–3 replicates. Translatability, measured by ribosome release from open reading frames after encountering a stop codon, is quantified in (B).

(C) Efficient and specific enrichment of mature lncRAP2 by hybridization-based purification. Exon-targeting probes effectively retrieve ~90% of cellular lncRAP2 RNA, quantified by qPCR, from differentiated 3T3-L1 adipocytes, whereas <1% was retrieved by intron-targeting probes, no probes, or probes targeting unrelated RNAs. RNase treatment eliminates lncRAP2 transcripts prior to purification. ***p < 0.001 (t test).

(D and E) ChIRP-MS identifies specific lncRAP2-binding proteins. The relative enrichment of high-confidence interactors captured by antisense purification of lncRAP2 in white adipocytes or other lncRNAs in other formaldehyde cross-linked cells is shown in (D). Unique peptide counts for specific lncRAP2 interactors are shown in (E).

(F) Validation of lncRAP2 and Igf2bp2 direct interaction in mature white adipocytes from mouse (left) and human (right). Native immunoprecipitation of Igf2bp2 specifically captures >50% of lncRAP2, compared to <2% for Ddx47 and Exosc6 (specific interactors) or Serbp1 and HnrpU (broad interactors). *p < 0.05 (t test).

(G and H) Igf2bp2 depletion blocks adipogenesis. Shown are lipid accumulation (G) and relative expression of key adipocyte genes (H) in day 6 differentiated white adipocytes pretreated with control or Igf2bp2-targeting DsiRNAs.
Figure 3. IncRAP2-Igf2bp2 complex regulates expression of target mRNAs encoding metabolically important proteins to potentiate adipocyte energy expenditure

(A and B) Igf2bp2 selectively binds mRNAs encoding adipogenic regulator and effector proteins in mouse white adipocytes. Shown is a gene set enrichment analysis highlighting significantly enriched (p < 0.05) gene sets and their associated biological processes (A), with member genes shown to the right, and specific enrichment of mRNAs encoding key energy metabolism controllers (B), from native Igf2bp2 immunoprecipitations. **p <0.01, ***p <0.001 (t-test).

(C) Depleting IncRAP2 or Igf2bp2 in mature adipocytes does not alter expression of adipose marker genes or each other’s RNA levels. Relative expression of key adipocyte genes in day 6 differentiated white adipocytes pretreated at differentiation day 4 with control, Igf2bp2-targeting (top), or IncRAP2-targeting (bottom) DsiRNAs.

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Among lncRAP2 interactors, Igf2bp2 regulates adipocyte function (Crat, Cpt2, and Bckdha) as well as regulators of mRNA translation and decay (e.g. Igf2bp2, Exosc6, and Ddx47).

In an unbiased approach, native immunoprecipitation of endogenous Igf2bp2 proteins detected by at least 2 unique peptides. We found 29 of these to be high-confidence (>2-fold enriched) lncRAP2-binding proteins (Data S1). To identify specific lncRAP2 interactors, we compared these proteins to those captured by the hybridization-based purification of other lncRNAs: Xist, Neat1, Malat1, H19, Paupar, and Bloodlinc (Alvarez-Dominguez et al., 2017a; Chu et al., 2015; Schmidt et al., 2018; Singer et al., 2019; West et al., 2014) in formaldehyde cross-linked cells. Notably, 26 out of the 29 interactors (90%) are lncRAP2-specific (Figures 2D and 2E), and include regulators of fatty acid and keto acid metabolism (Crat, Cpt2, and Bckdha) as well as regulators of mRNA translation and decay (e.g. Igf2bp2, Exosc6, and Ddx47).

IncRAP2 and Igf2bp2 stabilize target mRNAs encoding metabolic effectors to potentiate adipocyte energy expenditure

Igf2bp2, like IncRAP2, is highly expressed in white adipocytes (Figure S2F). We find little Igf2bp2 induction with adipogenesis, however, as it is already present in pre-adipocytes (Figures S2G and S2H). Depleting Igf2bp2 by ~50% with DsiRNAs in white preadipocytes blocks their subsequent differentiation, analogous to IncRAP2 inhibition, blunting induction of adipogenic markers, and lipogenesis (Figures 2G and 2H). The induction of IncRAP2 is also disrupted, consistent with a block in differentiation.

To investigate how Igf2bp2 regulates adipocyte function, we sought to identify its client mRNAs. Unbiased sequencing of RNAs captured by native Igf2bp2-specific immunoprecipitates reveals 1,657 substrates enriched over input RNA or control purifications with IgG or with no antibody (Figure S3A; Data S2). These comprise ncRNAs and mRNAs linked to various functions, with selective enrichment for adipogenic effectors (e.g. Nfat and Elovl proteins) and regulators (e.g. Cbp, Cited2, Ebf, and Klf factors) (Figure 3A). In line with known Igf2bp2 binding bias (Conway et al., 2016; Hafner et al., 2010; Ray et al., 2013), >60% of Igf2bp2 targets in adipocytes harbor CA-rich binding motifs, mainly enriched in 3'UTRs (Figure S3B), including several motif instances within IncRAP2's terminal exon (Figure S3C). Of 1,477 Igf2bp2 substrates with human homologs, 1,095 (~74%) also copurify with Igf2bp2 in human cell types cross-linked with UV (Van Nostrand et al., 2016) or 4-thiouridine (Hafner et al., 2010) (Figure S3D). Notably, conserved substrates include mRNAs encoding key controllers of energy metabolism, such as the namesake Igf1/2 targets and PPARγ coactivator 1α (PGC1α) (Figure S3B).
Igf2bp2 client RNAs that are induced or suppressed with adipogenesis are reciprocally regulated if lncRAP2 is depleted before differentiation (Sun et al., 2013) (Figure S3E). This could reflect Igf2bp2 and lncRAP2 sharing a common set of RNA targets, or merely the fact that both affect adipogenesis. To distinguish between these possibilities, we depleted lncRAP2 and Igf2bp2 in mature adipocytes, which allow studying the lncRAP2-Igf2bp2 connection independent of effects on adipogenesis. Transducing DsiRNAs against lncRAP2 or Igf2bp2 into differentiated white adipocytes deplete their target by 70%–80% without impacting each other’s levels (Figure 3C). Remarkably, unbiased RNA sequencing reveals that the RNA changes occurring after lncRAP2 depletion are tightly correlated with those occurring after Igf2bp2 depletion (Pearson’s r = 0.75, p < 10^-15, t test) (Figures 3D and S3F). By contrast, we find no correlation (Pearson’s r ~0) between the global RNA effects of depleting lncRAP2 or Igf2bp2 with those of depleting Ppary or linc-ADAL (lincRNA for adipogenesis and lipogenesis) in mature white adipocytes (Schupp et al., 2009; Zhang et al., 2018) (Figure S3H). Inhibiting either Igf2bp2 or lncRAP2 results in destabilization of the namesake Igf1/2 mRNA targets of Igf2bp2, as well as derepression of target mRNAs encoding thermogenic factors Ebf2 and PGC1α (Figure 3E). By contrast, non-target mRNAs encoding key adipogenic regulators (PPARY, C/EBPα) remain unaffected (Figure 3C).

Igf2bp2 client mRNAs encoding effectors of lipid/glucose synthesis and metabolism are selectively destabilized when either lncRAP2 or Igf2bp2 is inhibited (Figure S3G), suggesting that the lncRAP2-Igf2bp2 complex normally potentiates energy expenditure. To test this, we measured lipolysis in differentiated lncRAP2- and Igf2bp2-depleted adipocytes. In both cases, isoproterenol-induced lipolytic responses were reduced, and lncRAP2 depletion also lowered basal lipolysis (Figure 3H). Further supporting key roles in lipid metabolism, the gene signature of lncRAP2-Igf2bp2 depletion is most significantly associated with that of lysosomal acid lipase deficiency in lysosomal acid lipase gene knockout mice (Lian et al., 2005) (Figure S3I). These findings indicate that lncRAP2-Igf2bp2 complexes support energy expenditure in mature adipocytes by binding to and stabilizing many mRNAs encoding metabolic effectors.

**lncRAP2 and Igf2bp2 predominantly modulate target mRNA levels, not their translation**

To investigate how lncRAP2 and Igf2bp2 regulate their mRNA targets, we studied their protein levels and mRNA translation efficiency. Global protein levels were examined by quantitative mass spectrometry using tandem mass tag labeling in mature adipocytes. In total, proteins for 1,273 genes were quantifiable (detected by at least 2 unique peptides), including 138 whose mRNAs were found to be direct Igf2bp2 targets by RIP-seq. For both direct Igf2bp2 targets and non-targets, the changes in protein levels occurring upon lncRAP2 depletion closely correspond to those occurring upon Igf2bp2 depletion (Pearson’s r = 0.78 and r = 0.73, p < 10^-15, t test) (Figure 3I). These results support the notion that an lncRAP2-Igf2bp2 complex regulates a common set of mRNA targets. The targets include proteins with roles in lipid/glucose metabolism and oxidation that are selectively destabilized when either lncRAP2 or Igf2bp2 is depleted (Figure S4A), such as adiponectin and Fabp4, which further decrease under isoproterenol-induced stimulation of lipolysis (Figure 3G). By contrast, Igf2bp2 protein abundance was unaltered by lncRAP2 depletion, consistent with RNA-level results indicating that lncRAP2 and Igf2bp2 do not act to regulate each other’s levels.

The closely corresponding RNA and protein changes after either lncRAP2 or Igf2bp2 are inhibited suggests that the lncRAP2-Igf2bp2 complex regulates its targets by tuning their mRNA and thereby protein level. To test this, we compared RNA and protein responses to lncRAP2 or Igf2bp2 depletion in mature adipocytes for 1,205 genes quantifiable by both RNA-seq and mass spectrometry. The correlation between the two types of responses was strong for direct Igf2bp2 targets with differentially regulated protein vs. non-targets, both after lncRAP2 depletion (Pearson’s r = 0.51 vs. r = 0.28, p < 10^{-3} vs. p < 10^{-15}, t test) and after Igf2bp2 depletion (Pearson’s r = 0.32 vs. r = 0.12, p < 10^{-3} vs. p < 10^{-3}, t test) (Figures 3J and S4B). Any scatter that might have indicated that a few targets are translationally regulated (changing in protein but not RNA level) closely resembled the scatter observed in parallel for non-targets. We also calculated mRNA translation efficiencies, by dividing the level of steady-state ribosome-protected RNA fragments by that of total RNA fragments measured in parallel in mature adipocytes (Reid et al., 2017). We found no significant difference in translation efficiency between direct Igf2bp2 targets and non-targets, whether down/up regulated at the protein level after lncRAP2/Igf2bp2 depletion (Figure 3J). In summary, we found no evidence countering the conclusion that lncRAP2-Igf2bp2 complexes mainly act to modulate mRNA levels for most targets, without directly affecting their translation.
Figure 4. IncRAP2-lgf2bp2 genetic and expression variability are associated with obesity-linked diabetes risk

(A) A genetic variant linked to higher body mass, fasting insulin, and insulin resistance in Chinese females (Wang et al., 2008) (rs2209972) maps to a IncRAP2 linkage disequilibrium and structural domain. Heatmap displays linkage disequilibrium (LD) for a population of Han Chinese in Beijing and Japanese in Tokyo (JPT + CHB) (International HapMap, 2003) from phased genotypes of 90 unrelated individuals (Barrett et al., 2005). Tracks below display human white adipocyte open chromatin and histone mark sequencing studies, and chromatin interactions involving CTCF and cohesin subunits (SMC1, RAD21) (Data S4).

(B) IncRAP2rs2209972 is specifically associated with body mass, insulin secretion, and type 2 diabetes. Phenome-wide association results between rs2209972 and 317 phenotypes across 16,278,030 individuals of various ancestries from the Type 2 Diabetes Knowledge Portal (http://www.type2diabetesgenetics.org/variantInfo/variantInfo/rs2209972). Only significant (p <0.05) associations are shown.
IncRAP2 and Igf2bp2 are associated with obesity-linked diabetes risk

Given IncRAP2’s role in regulating adipocyte development and function, we sought validation of its importance from human genetics. A genetic variant (rs2209972:C) associated with increased body mass index, higher fasting insulin levels, and insulin resistance in women with polycystic ovary syndrome in a Chinese population (Wang et al., 2008) maps to the IncRAP2 gene body (Figure 4A). While this variant has been associated with the nearby gene encoding insulin-degrading enzyme (IDE) (Wang et al., 2008), three lines of evidence confine its genetic association to the IncRAP2 locus. First, in the Chinese and in all other populations surveyed, rs2209972 is in strong linkage disequilibrium ($r^2 > 0.8$) with variants within IncRAP2, but not so with any other locus within 500kb, including the IDE gene (Figures 4A and 5A). Second, IncRAP2 is confined to a closed chromatin loop formed by interacting sites of the CTCF insulator that are co-bound by cohesin (Figure 4A), insulating the IncRAP2 and IDE genes from each other. Third, rs2209972 and linked variants physically contact IncRAP2 but no loci outside its insulated structural domain, as evidenced by chromatin conformation analysis (Figure S5B).

The pathogenesis of type 2 diabetes can involve obesity-linked adipose dysfunction (Ashcroft and Rorsman, 2012; Rosen and Spiegelman, 2006). To explore IncRAP2-Igf2bp2’s contribution, we studied their regulation in obesity and diabetes progression. Adipose tissue IncRAP2/Igf2bp2 levels are progressively suppressed in mice and in lean/overweight humans fed a high-fat diet ($p < 10^{-3}$ to $10^{-1.3}$, t test) (Figure 4E). Human adipose IncRAP2/Igf2bp2 levels decrease as body mass index increases (Figures 4D and 5E), and tend to be restored upon weight loss after bariatric surgery (Figure 4F). We also found that Igf2bp2 trends lower in adipocytes of leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) obese mice (Figure 5F), which model diabetes onset and progression (Kleinert et al., 2018). We specifically studied Igf2bp2 in advanced human diabetics, matched for body mass index, and found strong downregulation in both visceral and subcutaneous adipose tissue of insulin-resistant compared with insulin-sensitive patients ($p < 10^{-3}$ to $10^{-2}$, t test) (Figure 4G). Thus, both genetic and expression variation of IncRAP2-Igf2bp2 are associated with obesity-linked diabetes outcomes. We propose that this results from reduced levels of IncRAP2-Igf2bp2 complexes, which limits their ability to program adipocyte metabolism by stabilizing mRNAs that encode key energy expenditure proteins.

DISCUSSION

Obesity has become pandemic (Hales et al., 2018; Vardell, 2020), increasing the worldwide prevalence of type 2 diabetes (Organization, 2016). Anti-diabetic drugs targeting white fat have not been successful (Kusminski et al., 2016; Socco et al., 2014), highlighting limited insight into how adipocytes develop and...
function. We and others have shown that lncRNAs contribute to adipocyte lineage specification and specialization (Knoll et al., 2015; Sun and Lin, 2019). Elucidating specific mechanisms has been elusive, however, due to the poor conservation of lncRNA genes and to the scarcity of lncRNA-centric tools. In this study, we made three important contributions to understanding how lncRAP2, conserved between mice and humans, regulates adipocyte function. First, multiple lncRAP2 alleles are specifically associated with diabetes, obesity, and metabolic traits. Second, lncRAP2 forms complexes with the RNA-binding protein Igf2bp2. And third, lncRAP2-Igf2bp2 complexes regulate metabolism in mature adipocytes by stabilizing client mRNAs encoding effectors of energy metabolism.

Our data show that multiple genetic variants associated with propensity to type 2 diabetes are confined to an lncRAP2 linkage disequilibrium and chromatin interaction domain. Analyzing hundreds of phenotypes interrogated by genome-wide association studies reveals that these variants, mapping to the lncRAP2 gene body and transcription start and end sites, are specifically associated with body fat mass, insulin secretion, and type 2 diabetes, implicating lncRAP2 alleles in the development of obesity and diabetes. Supporting this notion, lncRAP2 is suppressed in the white fat of mice and humans consuming a high-fat diet, and in humans lncRAP2 levels decrease progressively with increasing obesity, whereas lncRAP2 levels are restored upon weight loss.

lncRAP2 is enriched in white relative to brown adipocytes and is critical for adipogenesis (Sun et al., 2013). Although many lncRNAs appear to be nuclear-enriched (Derrien et al., 2012), lncRAP2 is mainly cytoplasmic, yet by ribosome footprint profiling we find no evidence of productive lncRAP2 translation. Using RNA interactome analyses in intact cross-linked cells, we found that lncRAP2 does not directly bind other RNAs, but specifically interacts with metabolic enzymes and mRNA decay/translation modulators. Some of these appear to be transient or indirect interactions, which may be of importance in other tissues where lncRAP2 is expressed. Importantly, in both mouse and human adipocytes, we verified a strong, direct binding of lncRAP2 to Igf2bp2.

Igf2bp2 is of prime interest, as it harbors one of the first genetic variants associated by genome-wide association studies with type 2 diabetes (Diabetes Genetics Initiative of Broad Institute of et al., 2007; Scott et al., 2007; Zeggini et al., 2007). This variant, and others in strong linkage disequilibrium, are associated with lower Igf2bp2 levels in pancreatic islets (Greenwald et al., 2019), and with impaired insulin responses (Wood et al., 2017). Yet the variants are also associated with increased body fat (Pulit et al., 2019), with a stronger effect in type 2 diabetes (Akiyama et al., 2017). Intriguingly, Igf2bp2-null mice are resistant to diet-induced obesity and diabetes, though this is likely due to underdeveloped white and overactive brown fat (Dai et al., 2015)—a tissue that is much less prevalent in adult humans (Rosen and Spiegelman, 2014). How Igf2bp2 impacts diet-induced obesity and diabetes risk, and its roles in mature white fat, have thus remained unclear.

We find that, in mature white adipocytes, Igf2bp2 binds to mRNAs encoding key adipogenic regulators and regulates their stability, explaining why Igf2bp2 is required for adipogenesis in vitro and in vivo (Dai et al., 2015; Zhang et al., 2018). Igf2bp2 also binds to mRNAs encoding effectors of energy metabolism, including Elovl factors, Fabp4, and adiponectin, thereby linking Igf2bp2 to lipid synthesis, transport, and metabolism. Indeed, we find hindered lipolytic responses upon Igf2bp2 inhibition, suggesting reduced energy expenditure capacity. Notably, Igf2bp2 also binds to mRNAs encoding energy metabolism effectors in human cells, and Igf2bp2 suppression in visceral or subcutaneous fat correlates with insulin resistance in diabetic patients.

### Table 1. Reported variants and their associated traits

| Variant | Trait | p value | Effect direction | Odds ratio | Effect size | Sample size |
|---------|-------|---------|------------------|------------|-------------|-------------|
| rs2209972(C) | Type 2 diabetes | 6.1E-09 | Increased | 1.039 | 898,130 |
| | BMI | 0.00142 | Increased | 0.0132 | 191,764 |
| | Incremental insulin at 30 min OGTT | 0.00207 | Decreased | –0.0800 | 5,318 |
| rs967878(G) | Type 2 diabetes | 7.10E-10 | Increased | 1.039 | 898,130 |
| rs7086285(T) | Type 2 diabetes | 6.50E-10 | Increased | 1.039 | 898,130 |
| rs884526(G) | Type 2 diabetes | 6.60E-09 | Increased | 1.039 | 898,130 |
| | BMI | 0.00136 | Increased | 0.0133 | 191,764 |
Remarkably, Igf2bp2 binds >55% of cellular IncRAP2, but not as a client RNA. Instead, IncRAP2 and Igf2bp2 interact to program white adipocyte development and metabolism. Supporting this conclusion, we show that depleting either IncRAP2 or Igf2bp2 in mature adipocytes causes tightly corresponding changes in both transcriptome and proteome. mRNAs encoding energy metabolism effectors are selectively destabilized, in both cases limiting lipid breakdown. We thus expect that disrupting IncRAP2-Igf2bp2 complexes in mature adipose tissue will cause fat accumulation, increasing obesity, and thereby propensity for diabetes.

Mechanistically, changes in protein levels of Igf2bp2 client mRNAs mirror changes in their mRNA abundance, indicating that neither IncRAP2 nor Igf2bp2 directly affects mRNA translation. IncRAP2-Igf2bp2 complexes thus fine-tune energy metabolism primarily by modulating client mRNA stability. Although inhibiting Igf2bp2 phenocopies the molecular and physiological effects of IncRAP2 inhibition, both interact with additional proteins, such that these effects must reflect perturbation of many functions in addition to those exerted in partnership with each other.

Our characterization of a IncRAP2-Igf2bp2 interaction as a posttranscriptional regulatory program in adipocyte metabolism echoes the finding that the Airn and HIF1A-AS2 lncRNAs are functional Igf2bp2 cofactors in the developing brain (Mineo et al., 2016) and in cardiomyocytes (Hosen et al., 2018), respectively. Igf2bp2 thus appears to bind distinct lncRNAs to regulate distinct targets in diverse tissues. Given that IncRAP2 is adipose-specific and conserved in mice and in humans, it represents an attractive target to selectively modulate Igf2bp2 activity within fat tissue to treat or prevent the progression of obesity-linked diabetes.

Limitations of the study

The experimental and computational systems used in this study have limitations to consider. In our experiments designed to investigate the functional relationship between IncRAP2 and Igf2bp2, we did not consider whether IncRAP2 modulates the affinity of Igf2bp2 for client mRNAs. Although we identify a human IncRAP2 homolog and characterize its regulation, its specific functions were not tested. We demonstrate that polymorphisms genetically and structurally associated with human IncRAP2 are linked to metabolic diseases, but their impact on the expression or function of human IncRAP2/Igf2bp2 and their targets was not investigated.

STAR★METHODS

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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103680.

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AUTHOR CONTRIBUTIONS

J.R.A.-D., M.K., and S.W. performed experiments; J.R.A.-D., M.K., and H.F.L. designed the research, interpreted results, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-IGF2BP2        | Abcam  | Ab151463   |
| anti-DDX47          | Abcam  | Ab128204   |
| anti-Exosc6         | Abcam  | Ab50910    |
| anti-Serbp1         | Abcam  | Ab57285    |
| anti-HNRNPU         | Abcam  | Ab20666    |
| **Bacterial and virus strains** | | |
| One Shot™ TOP10 E.coli | ThermoFisher | C404010 |
| **Biological samples** | | |
| Total RNA from Subcutaneous Adipose Tissue, BMI <24.99 | Zen-Bio | RNA-T10-1 |
| Total RNA from Subcutaneous Adipose Tissue, BMI 25.0–29.99 | Zen-Bio | RNA-T10-2 |
| Total RNA from Subcutaneous Adipose Tissue, BMI >30.0 | Zen-Bio | RNA-T10-3 |
| Human MTC™ Panel I | Takara | 636742 |
| Human MTC™ Panel II | Takara | 636743 |
| Human Testis Total RNA | Takara | 636533 |
| Human Adipose Tissue Total RNA | Takara | 636558 |
| Mouse Total RNA Master Panel | Takara | 636644 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Paraformaldehyd     | Electron Microscopy Sciences | 15713 |
| TRIzol Reagent      | Thermo Fisher Scientific | 15596026 |
| Oil-red O           | Sigma-Aldrich | O1391 |
| Glutaraldehyde (25%) | Electron Microscopy Sciences | 16216 |
| Glycine             | Sigma-Aldrich | G7126-10MG |
| **Critical commercial assays** | | |
| Lipofectamine RNAI MAX | Invitrogen | LMRNA015 |
| Adipolysis Assay Kit | Cayman Chemical | 10009381 |
| miRNAeasy Kit       | Qiagen  | n/a        |
| Superscript II reverse transcriptase | Invitrogen | 18064-022 |
| Fast SYBR® Green Mastermix | Thermo Fisher | 4385610 |
| DC Protein Assay Kit II | Bio-Rad | 5000112 |
| ECL Plus Western Lightning Reagent | Perkin Elmer | NEL102 |
| FirstChoice RLM-Race Kit | Invitrogen | AM1700 |
| Gel Extraction Kit  | Qiagen  | n/a        |
| TopoTA Cloning      | Thermo Fisher Scientific | K4575J10 |
| PARIS Kit cell fractionation | Life Technologies | AM1921 |
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Deposited data       |        |            |
| RNA- and RIA-seq are deposited at GEO | GSE190047 |
| Proteomics raw data at MassIVE | MSV000088559 |
| Experimental models: Cell lines |        |            |
| 3T3-L1               | ATCC   | ATCC-CL-173|
| Experimental models: Organisms/strains |        |            |
| CS7BL/6J Wild-type mice, male and female mice, age 4–6 weeks | Jackson Laboratory | 000664 |

**Oligonucleotides**

All Oligonucleotides are listed in Table S1 | N/A | N/A |

**Software and algorithms**

- **Bowtie2**
  - Source: Langmead and Salzberg (2012)
  - Identifier: http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

- **DESeq**
  - Source: Anders and Huber (2010)
  - Identifier: N/A

- **MACS2**
  - Source: Zhang et al. (2008)
  - Identifier: N/A

- **Samtools**
  - Source: Li et al. (2009)
  - Identifier: http://samtools.sourceforge.net/

- **Fastqc**
  - Source: Andrews, 2010
  - Identifier: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

- **Fastx_clipper, fastx_trimmer**
  - Source: Hannon et al., 2010
  - Identifier: http://hannonlab.cshl.edu/fastx_toolkit/index.html

- **STAR v2.6.1**
  - Source: Dobin et al. (2013)
  - Identifier: https://github.com/alexdobin/STAR

- **ImageJ**
  - Source: Schneider et al., 2012
  - Identifier: https://imagej.nih.gov/ij/

- **BEDTools**
  - Source: Quinlan and Hall (2010)
  - Identifier: N/A

**Other**

- heat inactivated newborn calf serum (HI-NCBS) | Life Technologies | 26010-074 |
- heat inactivated fetal calf serum (FBS) | Sigma-Aldrich | F2442 |
- Collagenase A | Roche | 10103578001 |
- BSA | Sigma-Aldrich | A7906 |
- Insulin | Sigma-Aldrich | 11882 |
- Dexamethasone | Sigma-Aldrich | D4902 |
- 3-isobutyl-1-methylxanthine (IBMX) | Sigma-Aldrich | I5879 |
- Rosiglitazone | Cayman Chemical | 71742 |
- 3,3’,5-triiodo-L-thyronine (T3) | VWR | 100567-778 |
- Indomethacin | Sigma-Aldrich | I7378 |
- Isoproterenol | Sigma-Aldrich | I6504 |
- HALT protease and phosphatase Inhibitor | Thermo Fisher Scientific | 78442 |
- Protein A/G beads | Santa Cruz | sc-2003 |
- Biotin-XX, SSE | Thermo Fisher Scientific | B1606 |
- SUPERRaseln RNase Inhibitor | Life Technologies | AM2694 |
- MyOne Streptavidin C1 magnetic beads | Life Technologies | 65001 |

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to the lead contact, Marko Knoll (markoknoll@gmail.com).
Materials availability
This study did not generate new unique reagents.

Data and code availability
- RNA-seq and RIA-seq data have been deposited at GEO (GSE190047) and are publicly available as of the date of publication. Proteomic raw data are deposited at MassIVE (MSV000088559). Accession numbers are listed in the key resources table. Original Western blot images and microscopy data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
C57BL/6J mice were bred in house or purchased from Jackson Laboratories (stock # 000664). All mice were housed under a 12 h light/dark cycle at constant temperature (20°C). All procedures were performed according to protocols approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

METHOD DETAILS

Isolation of primary cells and tissues
6–8 male 2–4 week old mice were sacrificed by CO₂ asphyxiation and interscapular brown adipose tissue (BAT) and subcutaneous (inguinal) white adipose tissue (scWAT) was harvested into room temperature plain DMEM (Sigma, # 56499C). The fat pads were transferred into a well of a 6 well plate and minced with scissors for 5 min. Minced tissues were then transferred into a 50 mL conical tube with 3 mL Hank’s balanced salt solution (Gibco, # 14175-095) supplemented with 0.2% collagenase A (Roche, # 10103578001) and 2% BSA (Sigma-Aldrich, # A7906) using a 1 mL pipet tip with the tip cut off to allow aspiration of larger pieces. The tissues were incubated agitating (350rpm) and repeated vortexing every 5 min for 10 s at 37°C for 30 min or 20 min for scWAT. Following collagenase digestion, 10 mL room temperature plain DMEM was added and cells were filtered through a 70 μm mesh filter (Corning, # 352350). Mature adipocytes and the stromal vascular fraction (SVF) were separated by centrifugation at 700 g for 5 min. The supernatant was removed and the SVF resuspended in 10 mL room temperature plain DMEM followed by additional filtering through a 30 μm mesh filter (Miltenyi Biotec # 130-041-407) and subsequent centrifugation at 700 g for 5 min. The SVF from subcutaneous white fat pads (scWAT) of 8 mice were then resuspended in 10 mL DMEM supplemented with 10% heat inactivated new born calf serum (HI NCBS, LifeTechnologies/Gibco, # 26010-074) and plated on two 10 cm dishes (Corning, # 430293). The SVF from interscapular brown fat pads of 6–8 mice were then resuspended in 6 mL DMEM supplemented with 10% HI NCBS and plated on 3 wells of a six well plate (Corning, #3506). After 4 and 24 h, the medium was replaced by fresh, pre-warmed DMEM/10% HI NBCS at 37°C and with 5% CO₂. Cells were grown to confluence and then passaged no more than two times before seeding the pre-adipocytes for differentiation.

Cell culture
Pre-adipocytes derived from BAT were cultured to confluence and then subsequently overgrown for 4–6 additional days until growth arrested. The cells were then induced to differentiate by culturing them for two days in induction medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich F2442) and 850 nM insulin (Sigma-Aldrich #I1882), 0.5 μM dexamethasone (Sigma-Aldrich #D4902), 250 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich #I8797), 1 μM rosiglitazone (Cayman Chemical #71742), 1 nM 3,3,5-triiodo-L-thyronine (T₃, VWR # 100567-778) and 125 nM indomethacin (Sigma-Aldrich #I7378). Subsequently, the induction medium was replaced with DMEM supplemented with 10% FBS and 160 nM insulin and 1 nM T₃ for another two days. The cells were then cultured in DMEM 10% FBS and 1 nM T₃ until day 8 of differentiation, and the medium was replaced every other day. Pre-adipocytes from scWAT were cultured similar but the induction medium and following medium did not contain T₃.
Cell stimulation
Mature adipocyte cell layers were washed twice in plain pre-warmed DMEM and stimulated with 1 μM isoproterenol (Sigma-Aldrich I6504). After 6 h of stimulation, the cells were washed once with cold PBS and RNA was harvested using TRizol or QIAzol lysis reagent as described below. For immunoblotting, the cultures were harvested after stimulation with isoproterenol after washing with cold PBS on ice and adding 40 μL RIPA buffer per well of a 6 well plate or 100 μL RIPA buffer to a 10 cm dish.

siRNA transfection
Was performed as described (Isidor et al., 2016). Briefly, on day 4 of differentiation, adipocytes were transfected with 5 nM siRNA (Sigma-Aldrich) using 5 μL/mL Lipofectamine RNAiMAX diluted in Opti-MEM I Reduced Serum Medium (Life Technologies). The cultures were analyzed on day 6 of differentiation.

Glycerol release
Following addition of fresh medium, cells were stimulated with isoproterenol. Cell culture medium was collected after 24h of stimulation and stored at −20°C. Glycerol release was measured using the Adipolysis Assay Kit (Cayman Chemical, 10009381) following the instructions of the manufacturer.

Quantitative PCR
Total RNA was isolated from tissues or cells using TRizol or QIazol reagent (LifeTechnologies/Ambion) and a miRNAeasy kit (Qiagen). 300 ng were reverse transcribed using Superscript II reverse transcriptase (LifeTechnologies/Invitrogen) using random hexamers (LifeTechnologies/Invitrogen). The cDNA was diluted 1:10 and 2.5 μL for a 96 well plate or 1 μL for a 384 well plate were used for quantitative Real-time PCR. qPCR was carried out on an ABI7900HT Fast real-time PCR system (Applied Biosystems) and analyzed using the delta delta Ct method normalized to 18S if not stated otherwise. Results are shown as pooled data from 3–4 independent experiments displaying the average and SEM.

RNA sequencing analysis
Poly A+ RNA sequencing (TrueSeqStrandedPolyA) was performed on RNA samples using a Nextseq genome sequencer (Illumina). RNA-seq paired-end reads were aligned to the mouse genome (mm9; NCBI Build 37) using STAR v2.6.1 (Dobin et al., 2013) provided with a splice junctions database (NCBIM37.67) and with default parameters and “--sjdbScore 2”.mRNA sequencing was performed on three independent experiments. Differential analysis and counts per millions (cpm) were obtained using DESeq (Anders and Huber, 2010) as described (Alvarez-Dominguez et al., 2017a).

Immunoblotting
Lysates were centrifuged for 10 min at 13,000 g to remove debris, and NuPAGE sample buffer and reducing buffer (LifeTechnologies) were added after measuring and adjusting the samples for protein concentration (DC protein assay kit II, Bio-Rad). 2–20 μg protein per sample were separated for 2–4 h at 60–100 V using 8%–26 well NuPAGE Bis-Tris Midi gels in MOPS or MES buffer (LifeTechnologies) and in Criterion cells (Bio-Rad) using respective adapters (LifeTechnologies). Protein was wet transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore) in Criterion blotter cells (Bio-Rad) using 2 x NuPAGE Transfer buffer (LifeTechnologies) with 10% methanol for 25 min at 1 A. After blocking the membrane in filtered (Nalgene # 595-4520) TBS-T (50 mM Trisbase, 150 mM NaCl, 0.1% Tween 20 [Sigma-Aldrich #P1379]) with 3% BSA (BSA, Sigma-Aldrich # A9906) for 1 h, blots were incubated in primary antibody diluted in TBS-T 3% BSA sealed in hybridization bags and gently shaking at 4°C overnight, then washed three times for 10 min in TBS-T, incubated in secondary antibody (Cell Signaling Technologies) diluted in TBS-T 3% BSA gently shaking for one hour at room temperature, and then washed again three times for 10 min in TBS-T. Antibody binding was visualized using ECL Plus Western Lightning reagent (PerkinElmer NEL 102) and blots were exposed to film (Kodak BioMax MR Film, Carestream Health Inc # 8701302). Films were scanned without adjustments using an Epson scanner. All immunoblotting data shown were reproduced with almost identical results in at least one and typically two to three additional independent experiments.

Oil-red O staining of brown adipocyte cultures
Cells were washed in PBS and fixed in 3.7% formaldehyde solution for 1 h, followed by staining with Oil Red O for 1 h. Oil Red O was prepared by diluting a stock solution (0.5 g of Oil Red O (Sigma) in 100 mL of
isopropanol) with water (6:4) followed by filtration. After staining, plates were washed twice in water and photographed.

5' and 3' RACE
The 5' and 3' ends of IncRAP2 was determined using the FirstChoice RLM-Race Kit from Ambion following the manufacturers instructions. Primers were designed accordingly and can be found in Data S3. Resulting gel bands were excised from the gel and purified using the Gel Extraction kit (Qiagen), cloned into a TopoTA vector (Thermo Fisher Scientific) and sequenced using the M13 fwd and rev primers supplied by the kit.

Cell fractionation
To separate the nuclear from the cytoplasmic fraction differentiated 3T3-L1 adipocytes were harvested and 1 million cells were used to isolated the fractions using the PARIS kit (Life technologies) according to the manufacturers instructions. Separated fractions were then analyzed using Real-time PCR and gene specific primers.

Single molecule FISH
Single molecule RNA FISH and fluorescence microscopy were described previously (Alvarez-Dominguez et al., 2014). Briefly, antisense probes were designed to span the exons of IncRAP2 (Data S3) and coupled to Cy5. Probes were hybridized at 2ng/µl final concentration. The maximum projection of FISH image z-stacks in the DAPI channel was merged with the z-slice of maximum contrast in the DIC channel, and the composite was used to identify cells. Images in the Cy5 channel were compared to those in the GFP control channel to detect diffraction-limited spots representing RNA transcripts using fixed pixel intensity thresholds. For image presentation, enhanced contrast in the DAPI channel was used to emphasize nuclear counterstaining boundaries.

Riboseq analysis
Ribosome profiling sequencing reads were clipped to remove 3' linkers using fastx_clipper from the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html), discarding non-clipped reads or reads <25nt after linker clipping ("-l 25 -c" parameters), and

we usedfastx_trimmer to remove the first 5' nucleotide from each read, as it often reflects an untemplated addition during cDNA generation. Pre-processed reads were then aligned to the mouse genome (mm9; NCBI Build 37) using STAR v2.6.1 with default parameters and "--seedSearchStartLmax 20 --sjdbOverhang 39 --outSJfilterOverhangMin 30 8 8 --sjdbScore 2". To measure gene-level and region-level (CDS, intron, 5'UTR, and 3'UTR) expression from uniquely-aligned reads, we quantified read counts using HTseq-count. Only genes for which a read count could be obtained in each sample and replicate were retained, and counts were then normalized as counts per million mapped reads (cpm) using only reads from these genes.

Translation efficiency analysis
Gene-level translation efficiency was calculated as the ratio of normalized Ribo-seq read density (cpm) to the normalized RNA-seq read density (cpm).

Ribosome release analysis
We computed the ribosome release score using the RRS program (Guttman et al., 2013) (default parameters and "--n false""--f false"), which measures the expected ribosome release after encountering a stop codon by calculating the ratio of the number of Ribo-seq reads in the ORF to the number of Ribo-seq reads in the 3'UTR, and dividing it by the same ratio calculated for RNA-seq reads (Guttman et al., 2013). For mRNAs, we used the longest annotated CDS and its 3'UTR to calculate the RRS. For IncRNAs, 5' and -3'UTRs, the RRS was computed for ORFs in all three possible frames whose 3'UTR was defined as the region between the stop codon and the beginning of the next ORF (in any frame) using the FindORFs utility (Guttman et al., 2013) (default parameters and "--c false --u true"). Only transcripts with a non-zero RRS were considered for all analyses.
**RNAcoimmunoprecipitation (RIP)**

RNA coimmunoprecipitation was done as described (Rinn et al., 2007). Mature adipocytes were harvested using trypsin. 1 x 10^7 re-suspended in 2mL 1X PBS, then lysed in nuclear isolation buffer (2mL nuclear isolation buffer + 6mL water, premixed) for 20 min. Nuclei were pelleted by centrifugation at 2,500 g for 15 min. Supernatant was discarded and nuclei were re-supplemented in 1mL RIP buffer containing the HALT protease and phosphatase inhibitor (Thermo scientific) and split into two halves and mechanically sheared using a Dounce homogenizer with 20 strokes. Nuclear membrane and debris were pelleted by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant was pre-cleared by adding 30mM slurry of protein A/G beads (Santa Cruz, sc-2003) and incubation for 2 h at 4°C on a rotator. Beads were removed by centrifugation at 2,500 g for 1 min and 10% of the supernatant was removed to a new tube (10% input) and the rest was incubated with antibodies to Suz12 (6ug; abcam, ab12073), IgG (10ug; abcam, ab37415), Ezh2 (8ug; abcam, ab3748), hnRNPU (8ug; abcam, ab20666), CoREST (8ug, santa cruz, sc23449) or no antibody for 3h at 4°C on a rotator. Then 60mL slurry of protein A/G beads were added for 2h at 4°C on a rotator. Beads were pelleted by centrifugation at 2500 rpm for 30s and washed 3 times in 500mL RIP for 10 min each followed by one wash with 1X PBS. For the isolation of RNA, the beads were re-suspended in TRIzol after the last wash step and isolated according to the manufacturers instructions. The RNA pellet was re-suspended in 10mL dH2O and was directly used for reverse transcription using random hexamers and Superscript II (Invitrogen). Analysis was done by real-time PCR and sequencing.

**RNA interactome analysis followed by sequencing (RIA-seq)**

RNA FISH probes were biotinylated using Biotin-XX, SSE (Thermo Fisher). As controls, we also used probes against H19 (Data S3) and Bloodlinc (Alvarez-Dominguez et al., 2017a). RIA-seq was conducted as described (Chu et al., 2011; Kretz et al., 2013). Briefly, 40 million day 6 differentiated adipocytes (3T3-L1) cells were harvested and crosslinked with 1% Glutaraldehyde for 10min at room temperature on a shaker, and the reaction was quenched with 0.125M glycine for 5min. Cells were collected by spinning at 2000g for 5min, resuspended in 4mL ice-cold PBS, and aliquoted to 4 collection tubes followed by resuspension in Lysis Buffer supplemented with Halt Protease and phosphatase inhibitor cocktail (Thermo Fisher, 100X) and with SUPERaseIn RNase Inhibitor (Life Technologies). Lysates were immediately sonicated in a Bioruptor (Diagenode) at 4°C using highest settings with 30 s ON and 45 s OFF for 3-45 h. The sonicated samples were spun at 16100g for 10min at 4°C, and the supernatant was then transferred to a new tube, with 10% taken as input RNA control (in TRizol). Hybridization Buffer and 100mM of the probes were added to the supernatant and incubated at 37°C for 4h in a rotator. 100mM of pre-washed Dynabeads MyOne Streptavidin C1 magnetic beads (Life Technologies) were then added and incubated for 30 min at 37°C in a rotator, captured by magnets (Invitrogen), and washed with Wash Buffer for 5 min at 37°C for a total of five times. After the last wash, beads were resuspended in 1mL Wash Buffer and then lysed using TRizol.

**RIA-seq analysis**

Raw reads (40bp unpaired and non-strand-specific) were mapped to mm9 using bowtie2 (Langmead and Salzberg, 2012). Peak calling was performed on uniquely-mapped reads relative to input control using MACS (Zhang et al., 2008) with default parameters and “--bw = 300–mfold 10 30”. High-confidence peaks were identified based on several criteria. First, peak coverage was quantified using BEDTools (Quinlan and Hall, 2010), and only peaks with an average per-base coverage greater than 2 reads were considered. Then peaks whose read density enrichment was at least 10-fold greater than the background model were considered enriched. Finally, peaks in the experimental sample overlapping peaks in the input control were excluded from further analysis.

**Comprehensive identification of RNA-binding proteins by mass spectrometry (ChIRP-MS)**

ChIRP-MS was performed as described (Chu et al., 2015) with the biotinylated probes used for RIA-seq. Since the first capture using even and odd probes didn’t result in pull-downs, we used all 48 exon probes for pull-down and intron, Bloodlinc, H19 probes as well as no probe control and RNAse A treated samples as control. Briefly, 100 million day 6 differentiated adipocytes from 3T3-L1 cells were harvested and washed twice in PBS, crosslinked in 3% formaldehyde for 30min, then quenched with 0.125M glycine for 5min, and collected by spinning at 2000g for 5 min. Cells were lysed and sonicated for 2:30 h. Lysates were pre-cleared by incubating with 50mL washed magnetic beads at 37°C for 30min and retrieving them with magnets before proceeding to hybridization with probes overnight at 37°C in a rotator. The following day, beads...
were added and incubated for 30 min at 37°C in a rotator, then washed five times with Wash Buffer but not eluted as described in (Chu et al., 2015); instead, beads were boiled for 10min at 95°C in Laemmli Buffer, then beads and buffer were separated on a NuPAGE 4–12% Bis-Tris gel, followed by silver staining to identify differential bands. The whole gel lane was then excised, trypsinized, reduced, alkylated, and trypsinized at 37°C overnight. The resulting peptides were extracted, concentrated, and HPLC-purified. Effluents were analyzed using an Orbitrap Elite (ThermoFisher) mass spectrometer in nanospray configuration operated in data-dependent acquisition mode, where the 10 most abundant peptides detected using full scan mode with a resolution of 240,000 were subjected to daughter ion fragmentation in the linear ion trap. A running list of parent ions was tabulated to an exclusion list to increase the number of peptides analyzed throughout the chromatographic run.

**ChIRP-MS analysis**

Mass spectrometry fragmentation spectra were correlated against a custom peptide database, formed by downloading all RefSeq species-specific (mm9) entries, and against a database of common contaminants (keratin, trypsin, etc.) using Mascot (Matrix Science) 2.5.1 and the Sequest algorithm (Thermo). The resulting Mascot search results were uploaded into Scaffold (Proteome Software), and a minimum of two peptides and peptide threshold of 95% and protein threshold of 99% were used for identification of peptides and positive protein identifications. Proteins enriched in ChIRP-MS experiments were identified based on several criteria. First, only proteins identified by 2 or more unique peptides in the differentiated sample versus intron probes were considered enriched. Finally, proteins introduced during sample preparation and purification (e.g. streptavidin, albumin) were excluded from further analysis. To distinguish IncRAP2-specific interactors from proteins that generally bind RNA during its processing, we compared the proteins enriched by IncRAP2 with those enriched by Dleu2 (Alvarez-Dominguez et al., 2017a), Neat1 and Malat1 (West et al., 2014), and U1, U2, and Xist (Chu et al., 2015), which also filtered for identification by 2 or more unique peptides, for enrichment relative to their control sample greater than 3-fold, and for known contaminants. Proteins enriched by each RNA were ranked according to their total peptide counts, and rankings were compared.

**Silver staining**

After proteins were separated on an Acryl-amid gel (see Western blot), the gel was fixed in 40% EtOH with 10% HAc for 1 h then washed 2 x 20 min in 30% EtOH and once for 20 min in water. The gel was sensitized for 1 min in 0.02% Na2S2O3 following 3 x 20 s wash in water and 20 min incubation in cold (4°C) 0.1% AgNO3 solution. The gel was washed 3 x 20 s in water and developed in a new chamber in 3% Na2CO3 with 0.05% formaldehyde. As soon as bands became visible the reaction was stopped by a short wash in water and then incubation in 5% HAc. All lanes were cut out and processed (see ChIRP-MS analysis).

**Gene set and pathway enrichment analysis**

Gene lists were analyzed for enrichment of genes grouped by biological process ontology or by curated annotations from the Molecular signatures database with GSEA (Subramanian et al., 2005) using default parameters and “-metric log2_Ratio_of_Classes”.

**Motif enrichment analysis**

Genomic sequences from regions of interest (e.g. UTRs) were searched for matches to a database of TF recognition sites (Saint-Andre et al., 2016) for TFs expressed in the relevant cell type using FIMO (Grant et al., 2011) as described in (Alvarez-Dominguez et al., 2017b) with minor modifications: a Markov model of sequence nucleotide composition was used as the background model for motif matching (to normalize for biased distribution of individual letters in the examined sequences), and motifs with an odds ratio>2 and q-value<0.05 (Fisher’s exact test) relative to 10 randomly-shuffled controls were considered significantly enriched.

**Chromatin interaction analysis**

Processed ChIA-PET, Hi-C, and capture Hi-C datasets were downloaded and visualized in triangle heatmap mode using the UCSC genome browser or in arc mode using the Washington University Epigenome browser, with default normalization and resolution settings and fixed display values.
Additional bioinformatics methods

All sequencing reads were quality-checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Genome-wide read density maps were generated by MACS2 using the “–bdg” option, normalized by RSeQC (Wang et al., 2012) using the “normalize_bigwig.py” function, and visualized using BEDTools and the UCSC genome browser. Signal coverage and signal change surrounding regions of interest (e.g. DMRs, enhancer sites) were visualized using the rgs.plot R package (Shen et al., 2014). Data heatmaps were generated using the heatmap.2 function of the gplots R package (http://CRAN.R-project.org/package=gplots).

QUANTIFICATION AND STATISTICAL ANALYSIS

No statistical methods were used to predetermine sample size or remove outliers. The statistical difference between two sets of paired count data (e.g. motif matches in test vs. randomly-shuffled sequences) was assessed by a Fisher’s exact test using the fisher.test R implementation with default parameters. For unpaired data, a Shapiro-Wilk normality test was first performed using the shapiro.test R implementation with default parameters; for normally distributed data we then used a two-sided ttest (t.test R implementation with default parameters) to assess confidence on the measured difference of their mean values. For unpaired data that don’t follow a normal distribution, we used a non-parametric Wilcoxon rank-sum test to determine if they belong to the same distribution. Variance was represented as mean ± SEM of n = 3 replicates unless otherwise specified.

ADDITIONAL RESOURCES

Human IncRAP2 sequence
>hg19_chr10: 94,176,182-94,180,397

ATCCGTATCCCAAGGGCTGATCCAAAGGAGCCACGACGTGAACTTCACCAGCAATCGTTAAATACTGATCCGAAGAGGAGGGTCTTCTTTATT

GTTCCTTCAGGCAAACCGGCAGCTGAGTCACCTGTGGAACCTCTTTCTGGGCTGGCA

CCTGTTGGGCTCCGCGCCGGACGTACCTGTCCCATCGGGGGCTGGCA

CAGGGCCTGTTGGGCTGCCCGGCCGCGCGTACCTGTCCCATCGGGGGCTGGCA

GCCAGGAGACGGGGTGAGTGAGCCAGAGCCAGGGACCTGGGGGCTGGTG

GCCGGGAGACGGGGTGAGTGAGCCAGAGCCAGGGACCTGGGGGCTGGTG

CCCGCAGCGTTGGGCGCTGCTGGTGGTGGTGGAGGTCTGGCAAGGCCTGGGCTGGTGAATTGGCTGTGTTGCTGCCCTTCTGCCCTGTCTCCT

GATCCTGTTCAGACTCTGCGGTGGCTCCTTTGAGTGATCTTCAGGCCTGGA

AGATGGGAAAGGCAAGGAGGTGGTGGGCTGCCCGGCGCGCGAGAG

ACTCTGAGCTGTACCTGTGGGGCTGGGATGAAACCACTGCTGCTCCGGGCTGGCAGAG

ACCCTGAGCTGTACCTCCTGGGATGAAAGGAGAACCCAGGAGCAGGCAGG
GAAAGATAACAGGCTGGGTGTGGCTACGCCTGTAATTCCAGCACT
TGGGGAGCGGAGGTGGGCAATCTACCTGAGCTCGGGAGTGTGAGAAAAG
GTATATATGTTTTGGCTTTTTAAAGAGCCCACAATATCTACACTTTAAAAT
ATTTCATATTTTCTATTTAACTCTAAATGATTGTTTTTAAAAATGATGCCA
CAACCTAGCTGGACTATTATGATAGTGTATAAGTATGTCTTTGTTGTACGA
CACATAGACCTTCACTTACCCACCTGAGCTCTAGACTGACCTGAAGGATAGCT
TCATCATATTTTCTATTAAAGTTTTGCCCCATTTTCTATTGCAATGATT
CCCTTTTTCCATGAGCTGCTATGTCAGTCTCAGCATCTTTCAATGTAGAG
TTTGAGCTATGAGTGGAGAAAAGCACATTTTTCTACTTTTTAAATGGAAT
AATCCACTGTGCCTGTGTTAATTTGATGACACTCATCTATTTATCTAGT
ATCCAGTCACCAAAAGCCATTACACGCCCCCAAGATCCACACCCCTTT
TTCCAGTCACCTTTCTCGACGTGATACCAACTCTCTCTGTATTTTGACAAG
ATAGATCATTATTTGCTTTTATTTGACATGATTGATCACAG
TATGAGATCTTTGGTCTGGTTTTTGAATTTTTGTTTTGTTTTGTTTTTCTTGAGATCGTGCTCTTGTTGCCCAG
GCTGGAGTGCAATGGCACGATCTCAGATCACTGCAACCTCCACCTCCTGG
GTTCAAGCAATTCTCCTGCAGCTCCTCAGCTCCAAGTAGCTGCTGGATTATAGGA
GCTTTGACCATTACCCACTTTGAGCTGCTCTTTAGTTGATTTTAGTGCAGTTG
CCTTTGACTCTTTCATTTTGCTTATTGTCAGTCTTCTGTTGCTCCCTG
GCCTACGCTGACGTCTGAGCTGCTTCTCACTGCTTGAGCTTCAGCTTGCTT
CTGCCATAAACATTCTTGTTCAGGGTTTTGGGGGACATATATATGGCTTT
CTGTTGGATATTTGAGATCAGTATTGTCAGCTGCTTTGAGATTTTAGTTT
AAATGCTCATTTGTGCTGACTGAACAAAAATTTTATTTTTATTTTATTTATCT
TTACTGTTACTGGGACGTGAGTTCTCGTTCACTTGTTTACGATGCTATAGTG
CTGCCTAAACATTCTTGTTCAGGTTTTTGGGGGACATATATTGGCTTT
CTGTGAGATATATATATAATTTAAGGGTGTTGCTGAAACACATTTTTGA
CAGTTTATGCTAACAAGGTGACTCTGTGTTAGGCCCTTTAGGCGGATGAT
ATCAGCCTGACCTCCAGAGATGGGGGCTGGAGACTGAGTTCAAC
CACATGGACAAATAAGTCTCATGTAATGAAAGCCCCAGTAAAAACTCTGG
ATGCTGAAGGTCAGGTGAATCCCTGATTGCAGTACTCTATATGTGT
GTCTCACACATCAAATCAGCAGGGGAATGCAATCTGAGGACCCCAGAGG
CTTCACATTTGGAACCTCTCTAGACTCTGCTCTATCCATCTCTTTTTT
GCTAATTTGTATCTCCTCTCCCTGAAAATGTGTGAGTA
TAACAG.