IGF2BP2 binds to a number of RNA transcripts and has been suggested to function as a tumor promoter, although little is known regarding the mechanisms that regulate its roles in RNA metabolism. Here we demonstrate that IGF2BP2 binds to the 3′ untranslated region of the transcript encoding ATP6V1A, a catalytic subunit of the vacuolar ATPase (v-ATPase), and serves as a substrate for the NAD⁺-dependent deacetylase SIRT1, which regulates how IGF2BP2 affects the stability of the ATP6V1A transcript. When sufficient levels of SIRT1 are expressed, it catalyzes the deacetylation of IGF2BP2, which can bind to the ATP6V1A transcript but does not mediate its degradation. However, when SIRT1 expression is low, the acetylated form of IGF2BP2 accumulates, and upon binding to the ATP6V1A transcript recruits the XRNU nuclease, which catalyzes transcript degradation. Thus, the stability of the ATP6V1A transcript is significantly compromised in breast cancer cells when SIRT1 expression is low or knocked-down. This leads to a reduction in the expression of functional v-ATPase complexes in cancer cells and to an impairment in their lysosomal activity, resulting in the production of a cellular secretome consisting of increased numbers of exosomes enriched in ubiquitinated protein cargo and soluble hydrolases, including cathepsins, that together combine to promote tumor cell survival and invasiveness. These findings describe a previously unrecognized role for IGF2BP2 in mediating the degradation of a messenger RNA transcript essential for lysosomal function and highlight how its sirtuin-regulated acetylation state can have significant biological and disease consequences.

Significance

Insulin-like growth factor 2 messenger RNA binding protein 2 (IGF2BP2) has been reported to bind and stabilize a variety of RNA transcripts. However, we now show that depending upon its acetylation status, IGF2BP2 helps to mediate the degradation of a transcript that has important consequences for breast cancer progression. The acetylation of IGF2BP2 is regulated by sirtuin 1 (SIRT1), such that SIRT1 downregulation in aggressive breast cancers increases the acetylated form of IGF2BP2, which binds and promotes the XRNU-mediated degradation of the RNA transcript encoding ATP6V1A, a catalytic subunit of the vacuolar ATPase (v-ATPase), thus impairing lysosomal function and resulting in a secretome that greatly enhances cancer cell invasiveness.
Given these findings, we set out to determine whether there might be a connection between SIRT1 down-regulation in breast cancer and the production of extracellular vesicles which have been linked to a number of aspects of tumorigenesis (16–19). This led us to discover that the reduced expression of SIRT1 in aggressive breast cancer cells was accompanied by an increased production of exosomes, a class of extracellular vesicles that form as intraluminal vesicles within multivesicular bodies during endosomal/lysosomal trafficking and are then shed from cells when multivesicular bodies fuse with the plasma membrane (20). Moreover, we found that knocking down SIRT1 in breast cancer cells also secreted increased amounts of cathepsins, which, together with the enhanced shedding of exosomes enriched in ubiquitinated protein cargo, produced a secretome that promoted cancer cell survival and invasiveness. We further determined that these effects accompanying SIRT1 down-regulation compromised lysosomal function because of a reduced expression of the ATP6V1A subunit of the vacuolar ATPase (v-ATPase). This then raised an important question: How does a reduction in SIRT1 expression give rise to a corresponding decrease in the levels of ATP6V1A? Here we show that in breast cancer cells where SIRT1 levels are low there is an increase in the acetylated form of IGF2BP2, which by specifically recruiting a nuclease promotes the degradation of the ATP6V1A transcript and reduces the expression of a major catalytic subunit of the v-ATPase. The end result is an impairment of lysosomal function in a manner that produces a unique secretome which helps contribute to the progression of aggressive breast cancers.

Results

SIRT1 Down-Regulation Reduces the Levels of the RNA Transcript Encoding the ATP6V1A Subunit of the v-ATPase. In order to establish that the reductions in ATP6V1A expression we previously observed were not unique to SIRT1 knock-down cells but also occurred in cancer cells with low endogenous SIRT1 expression, we examined the MCF10A breast cancer progression series which consists of isogenic cell lines that represent different stages of breast cancer, ranging from nonmalignant MCF10A epithelial cells to the highly malignant and aggressive MCF10CA1A cell line (21). The lowest levels of SIRT1 and ATP6V1A expression were detected in the malignant MCF10CA1A cells (SI Appendix, Fig. S1A), which was accompanied by an increase in the number of shed exosomes (SI Appendix, Fig. S1B). These results corroborate our earlier findings from SIRT1 knock-down cells and are consistent with data from the Cancer RNA-Nexus database, comparing triple-negative breast cancers (TNBCs) to normal tissues adjacent to TNBC tumors, which shows that more than 80% of the tumor samples exhibited a marked reduction in both SIRT1 and ATP6V1A transcript levels (20).

We then probed the consequences of disabling SIRT1 on exosome production in a mouse model for breast cancer by treating tumor-bearing MMTV-PYMT mice (22) with EX-527, a small-molecule inhibitor of SIRT1 deacetylase activity (23). Treatment with the SIRT1 inhibitor reduced ATP6V1A expression in tumor lysates (SI Appendix, Fig. S1C), and the exosomes isolated from their pooled serum, as detected using the exosomal markers CD9 (24) and Flotillin-2 (20), were free of cytosolic contaminants such as α-tubulin (SI Appendix, Fig. S1D) but were enriched in ubiquitinated proteins, compared to exosomes obtained from the serum of control mice (SI Appendix, Fig. S1E). Similar increases in ubiquitinated protein levels were also observed in exosomes isolated from the serum of mice bearing tumors of MDA-MB-231 breast cancer cells in which SIRT1 had been knocked-down (SI Appendix, Fig. S1F). Among the proteins enriched in exosomes isolated from breast cancer cells depleted of SIRT1 is Survivin, which has been shown to be essential for cancer cell survival, migration, and invasive activity (20; also see ref. 20 for a more complete list of proteins enriched in exosomes from SIRT1 knock-down cells).

IGF2BP2 Binds to the ATP6V1A Transcript and Mediates Its Degradation in SIRT1 Knock-Down Cells. An important and unanswered question concerned the mechanistic basis for how SIRT1 regulates the expression of the ATP6V1A transcript. Our earlier work suggested the reduction in ATP6V1A expression that occurred when knocking down SIRT1, for example as shown in SI Appendix, Fig. S2A using two different short hairpin RNAs (shRNAs) that target SIRT1, was not caused by an inhibitory effect on transcription (20). However, a significant clue to the underlying mechanism emerged when we discovered that treating cells with EX-527 to inhibit SIRT1 deacetylation activity resulted in an accelerated decay of the RNA transcript encoding ATP6V1A (Fig. 1A). We then found that the high ectopic expression of an ATP6V1A transcript which contained the coding sequence (CDS) but lacked the 3′ untranslatable region (3′ UTR) (Fig. 1B, Top) was not degraded under conditions where SIRT1 expression was knocked-down (Fig. 1B, Bottom Left), unlike the case when the 3′ UTR was present which resulted in ATP6V1A protein levels being significantly reduced (Fig. 1B, Bottom Right). Thus, when taken together these results identified a regulatory role for SIRT1 involving the 3′ UTR of the ATP6V1A transcript, which is necessary for maintaining the stability of this transcript.

In order to further pursue this lead, a biotinylated form of the 3′ UTR of the ATP6V1A transcript was generated and incubated with extracts collected from SIRT1 knock-down cells (SI Appendix, Fig. S2B). The biotinylated 3′ UTR construct was precipitated using streptavidin-coated beads and proteins that associated with the construct were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with Colloidal Blue. The Colloidal Blue-stained bands from two regions of the gel corresponding to approximate molecular masses of 80 kDa and 60 to 65 kDa (SI Appendix, Fig. S2C, boxed in red) were excised and analyzed by mass spectrometry. Among the 3′ UTR-associated proteins identified from the excised bands were several RNA-binding proteins including ILF3, FXR1, and FXR2 (25–27), as well as IGF2BP2 (SI Appendix, Fig. S2D). A complete list of the proteins identified can be found in the ProteomeXchange Consortium via the PRIDE partner repository using the identifier PXD028084. When each of these proteins was knocked-down in cells lacking SIRT1, only the knockdown of IGF2BP2 (Fig. 1C, Top) significantly restored ATP6V1A transcript levels (SI Appendix, Fig. S2E and Fig. 1D) and protein expression (Fig. 1C, Top). This demonstrated that among these RNA-binding proteins IGF2BP2 is specifically responsible for mediating the degradation of the ATP6V1A transcript.

Using quantitative RT-qPCR to compare the relative amounts of ATP6V1A transcript that communoprecipitated with Flag-tagged IGF2BP2 versus Flag-tagged Quaking 5 (QKI5), an RNA-binding protein whose homolog in Caenorhabditis elegans was reported to regulate ATP6V1A (vha-13) expression (28), we found that IGF2BP2 was much more effective than QKI5 in its ability to bind to the transcript (Fig. 1E). Enhanced crosslinking communoprecipitation analysis (29) for potential IGF2BP2 binding sites on the ATP6V1A transcript highlighted a number of possible points of contact, especially within the 3′ UTR
This was corroborated by an experiment showing that IGF2BP2 binds much better to an ATP6V1A transcript consisting of both its coding region and 3′ UTR (CDS + 3′ UTR), compared to the CDS alone (SI Appendix, Fig. S3B). In vitro RNA pull-down assays using a series of truncations of the ATP6V1A 3′ UTR demonstrated that IGF2BP2 binds with highest affinity to the full-length (2,631 base pairs) 3′ UTR (Fig. 1F, the T1 construct), while still maintaining some capability for binding to a 3′ UTR construct consisting of only the first 824 base pairs (i.e., the T3 construct). As previously reported, we observed that IGF2BP2 has multiple translation initiation sites (30), resulting in two predominant bands being detected by Western blot analysis (Fig. 1F, Bottom).

**Knockdowns of IGF2BP2 Reversed the Effects of Knocking Down SIRT1 on Exosome Production, Cathepsin Secretion, and Invasion.** Knocking down IGF2BP2 in cells depleted of SIRT1 decreased the amount of exosomes they produced, such that

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**Fig. 1.** The effect of depleting cells of SIRT1 on ATP6V1A expression levels is mediated by IGF2BP2. (A) ATP6V1A mRNA transcript stability assays were performed on Actinomycin A–treated MDA-MB-231 cells treated without (dimethyl sulfoxide, DMSO) or with EX-527 for the indicated times. The results are presented as the expression levels determined for the ATP6V1A transcript relative to actin transcript levels. (B) Representative immunoblots of whole-cell lysates (WCLs) from control (CTRL) and SIRT1 knockdown (SIRT1 KD) MDA-MB-231 cells ectopically expressing plasmids that encode the empty vector, the coding region of the ATP6V1A (CDS), or the coding region together with its 3′ UTR (CDS + 3′ UTR), probed for ATP6V1A, SIRT1, and β-actin as a loading control. (C) Representative immunoblots of WCLs from control, SIRT1 knockdown (KD), and SIRT1/IGF2BP2 double-KD MDA-MB-231 cells probed for ATP6V1A, SIRT1, IGF2BP2, and β-actin as a loading control (Top). Quantification of ATP6V1A protein levels (relative to actin levels) in these experiments (Bottom). (D) RT-qPCR was performed on the cells described in C to determine the relative expression levels of the ATP6V1A transcript (relative to actin transcript levels). (E) Representative immunoblots of WCLs from MDA-MB-231 cells ectopically expressing FLAG-tagged ATP6V1A and QKI5 probed for the FLAG-tagged proteins and β-actin as a loading control (Left). RT-qPCR was performed on the immunoprecipitated FLAG-tagged proteins to determine the relative levels of the ATP6V1A transcript that associated with each construct (Right). (F) Streptavidin pull-down assays performed on MDA-MB-231 cell WCLs incubated with the indicated biotinylated segments of the 3′ UTR of the ATP6V1A transcript (diagram, Top) were probed for IGF2BP2 (blot, Bottom). The relative expression levels of the indicated proteins, relative to controls, in B, C, and F were quantified based on densitometry and shown above the bands. The data shown in A, C, and D are presented as mean ± SD. Statistical significance in A and F was determined using Student’s t test and in C and D using a one-way ANOVA test; *P < 0.05, **P < 0.001; ns, not significant.
the numbers of shed vesicles were similar to those for control cells (Fig. 2 A and B). The double knockdown of SIRT1 and IGF2BP2 also eliminated the enrichment in ubiquitinated exosome cargo proteins and decreased the secretion of soluble hydrolases such as Cathepsin B (Fig. 2 C and D). We then examined the effects of depleting SIRT1 and IGF2BP2 on the invasive activity of MDA-MB-231 cells following their transplantation into the mammary fat pads of mice. Knocking down SIRT1 in MDA-MB-231 mouse xenografts caused a significant increase in tissue compartmental invasiveness (i.e., into skeletal muscle; Fig. 2 E and F), consistent with the findings from our earlier three-dimensional culture experiments which showed that depleting SIRT1 increased the invasive activity of breast cancer cells (20). This effect was then reversed in animals when both SIRT1 and IGF2BP2 were knocked down.

IGF2BP2 Is a Substrate for the SIRT1 Deacetylase. Our data showing that IGF2BP2 is responsible for ATP6V1A transcript degradation in the absence of SIRT1 led us to examine whether IGF2BP2 is a deacetylation substrate for SIRT1 and whether its ability to degrade ATP6V1A is regulated by acetylation. When acetylated proteins were immunoprecipitated from SIRT1 knockdown cells using an antibody that detects acetylated lysine moieties, there was an obvious increase in the amount of endogenous IGF2BP2 detected in the immunoprecipitates (Fig. 3 A). The same was true for p53, a known SIRT1 substrate (31), while the amount of acetylated tubulin was unaffected by knocking down SIRT1 (32). We then immunoprecipitated ectopically expressed FLAG-tagged IGF2BP2 from SIRT1 knock-down cells and identified lysine residue 530 as an acetylation site by mass spectrometry (Fig. 3 B). Western blot analysis performed on the same

Fig. 2. Depletion of IGF2BP2 rescues the effects caused by SIRT1 down-regulation. (A) Nanoparticle tracking analysis (NTA) was performed on the conditioned media collected from an equivalent number of control (CTRL), SIRT1 KD, and SIRT1/IGF2BP2 double-KD MDA-MB-231 cells. (B) Quantification of the assay shown in A. (C) Representative immunoblots of exosome lysates (EXO) prepared from control, SIRT1 KD, and SIRT1/IGF2BP2 double-KD MDA-MB-231 cells probed for ubiquitinated proteins and HSP90 as a loading control. (D) Representative immunoblots of vesicle free medium (VFM) prepared from the cells described in C probed for Cathepsin B and HSP90 as a loading control. (E) Images of representative tumor sections showing invasive fronts for tumors that formed in mice injected with an equivalent number of control (Ad), tumor (T), and invasive nodules (Inv n) are indicated. (Scale bar, 250 μm.) (F) Quantification of the assay shown in E (n = 8 for control cells, n = 7 for SIRT1 KD cells, and n = 6 for SIRT1/IGF2BP2 double-KD cells). The data shown in B and F represent means ± SD. Statistical significance was determined using a one-way ANOVA test; *P < 0.05 and ns, not significant.
immunoprecipitated samples using an acetylated lysine antibody showed that the acetylation of FLAG-tagged IGF2BP2 was increased when SIRT1 expression was knocked down (Fig. 3C, Top, compare lanes 2 and 4). Changing lysine 530 to an arginine residue (IGF2BP2(K530R)) significantly decreased the levels of acetylated FLAG-tagged IGF2BP2 detected in SIRT1 knockdown cells (Fig. 3C, Top, compare lanes 4 and 5). To further confirm that SIRT1 deacetylates IGF2BP2, FLAG-tagged forms of IGF2BP2 and the IGF2BP2(K530R) mutant were immunoprecipitated from SIRT1-depleted cells and then incubated with purified recombinant SIRT1. While the acetylation of IGF2BP2 (wild type, WT) was clearly reduced after incubation with recombinant SIRT1, the acetylation level of the K530R mutant was barely detectable and was completely unaffected by treatment with recombinant SIRT1 (Fig. 3D). We then examined whether an acetylation-defective form of IGF2BP2, when ectopically expressed, acted as a dominant-negative competitor of endogenous WT IGF2BP2 by blocking its binding to the ATP6V1A transcript and preventing transcript degradation that accompanied knockdowns of SIRT1. Indeed, ectopic expression of the acetylation-defective FLAG-tagged IGF2BP2(K530R) mutant eliminated most of the reduction in ATP6V1A expression that occurred in SIRT1-depleted cells (Fig. 3E, Right), compared to when ectopically expressing WT IGF2BP2 in SIRT1 knockdown cells (Fig. 3E, Left).

Lysine 530 is located within the KH4 domain of IGF2BP2, the last of its six RNA-binding domains which includes RRM1, RRM2, and KH1–4 (SI Appendix, Fig. S4A) (1–4). Alignment of the X-ray crystal structure of IGF2BP2 (33) with that for the highly related zinc-binding protein 1 (ZBP1) complexed to a segment of RNA (34) shows that lysine 530 is in the vicinity of a portion of the bound RNA (SI Appendix, Fig. S4B). Therefore, we first determined whether acetylation of lysine 530 in IGF2BP2 affected its ability to bind the ATP6V1A transcript. Immunoprecipitation assays were performed to compare the relative amounts of ATP6V1A transcript that coprecipitated with ectopically expressed Flag-tagged WT IGF2BP2, versus either Flag-tagged constructs of an acetylation-defective mutant (i.e., IGF2BP2 K530R) or a truncated IGF2BP2 protein lacking the KH4 domain. However, as shown in Fig. 3F, neither the acetylation of lysine 530 nor the truncation of the KH4 domain significantly altered the relative amount of ATP6V1A transcript that associated with IGF2BP2.

Acetylated IGF2BP2 Recruits the Nuclease XRN2 to Degrade the ATP6V1A Transcript. Since acetylation of IGF2BP2 was not an essential determinant for binding the RNA transcript encoding ATP6V1A, it seemed unlikely that IGF2BP2 alone accounted for its degradation. We therefore examined whether IGF2BP2 acetylation recruited an additional protein(s) that might be responsible for degrading the RNA transcript. Mass spectrometry was used to identify proteins that coimmunoprecipitated with Flag-tagged IGF2BP2 in SIRT1-depleted cells. This yielded several potential IGF2BP2 binding partners with the 5′-3′ exonuclease 2 (XRN2) and Y-box containing protein 1 (YBX1) having the highest number of peptide-spectrum matches (35, 36) (Fig. 4A). A complete list of the proteins identified can be found in the ProteomeXchange Consortium via the PRIDE partner repository using the identifier PXD028114. In vitro RNA pull-down assays showed that XRN2 associated with the ATP6V1A 3′UTR (SI Appendix, Fig. S5A). We also found that the ability of XRN2 to bind ectopically expressed FLAG-tagged IGF2BP2 as detected by their coimmunoprecipitation was significantly enhanced in SIRT1 knock-down cells (Fig. 4B, top row), suggesting a preferential binding to acetylated IGF2BP2, whereas the binding of YBX1 to IGF2BP2 was unaffected (Fig. 4B, second row). Moreover, the ability of XRN2 to bind IGF2BP2 was weakened upon changing lysine 530 to arginine and eliminated when the KH4 domain was deleted (Fig. 4C, Upper, top row, and 4D). Knocking down XRN2 and/or IGF2BP2 in cells depleted of SIRT1 resulted in an increase in the transcript levels of ATP6V1A and reacidified the lysosomes in cells lacking SIRT1 (Fig. 4E and SI Appendix, Fig. S5B) as well as reduced the number of exosomes shed by SIRT1-depleted cells (Fig. 4F and SI Appendix, Fig. S5C), together with the amount of ubiquitinated proteins found in their exosome cargo (Fig. 4G), and significantly decreased the levels of secreted Cathepsin B (SI Appendix, Fig. S5D). However, the ectopic expression of XRN2 in cells did not cause a reduction in ATP6V1A expression levels (SI Appendix, Fig. S5E), consistent with the idea that XRN2 needs to work together with IGF2BP2 to promote the degradation of the transcript.

XRN1 (35), a closely related family member to XRN2, was also capable of associating with IGF2BP2 when XRN2 was knocked down in cells depleted of SIRT1 (SI Appendix, Fig. S6A), although knocking down XRN1 together with SIRT1 failed to show a significant restoration of ATP6V1A transcript levels (SI Appendix, Fig. S6B, compare lanes 2 and 4). However, the double knockdown of XRN1 and XRN2 in cells depleted of SIRT1 restored ATP6V1A protein expression to even greater levels compared to when knocking down XRN2 alone (SI Appendix, Fig. S6B, compare lanes 3 and 5). These results suggest that XRN2 may be the dominant nuclease responsible for degrading the ATP6V1A transcript in cells where SIRT1 expression has been reduced, whereas the related XRN1 appears to be capable of providing some compensation when XRN2 has been knocked down.

Discussion

We have discovered that the regulation of the RNA-binding protein IGF2BP2, by acting as a substrate for the deacetylase SIRT1, has an important role in maintaining lysosomal function. Previous studies have implicated IGF2BP2 in various biological and disease contexts including different forms of cancer, by acting to stabilize RNA transcripts and modified forms of long noncoding RNAs (1–7, 37–39). However, we now show that the loss of a regulatory deacetylation of IGF2BP2, when depleting cells of SIRT1 activity, results in IGF2BP2 promoting the degradation of the RNA transcript that encodes ATP6V1A, a major catalytic subunit of the v-ATPase. These findings shed further light on how SIRT1 down-regulation contributes to tumor progression (13–15), by highlighting an unexpected role in RNA metabolism, as well as providing a mechanistic basis by which IGF2BP2 serves to promote invasive activity in breast cancer (40). Thus, when SIRT1 levels are down-regulated, the acetylated form of IGF2BP2 accumulates and recruits the exonuclease XRN2, thereby promoting the degradation of the RNA transcript that encodes ATP6V1A, a major catalytic subunit of the v-ATPase. These findings shed further light on how SIRT1 down-regulation contributes to tumor progression (13–15), by highlighting an unexpected role in RNA metabolism, as well as providing a mechanistic basis by which IGF2BP2 serves to promote invasive activity in breast cancer (40). Thus, when SIRT1 levels are down-regulated, the acetylated form of IGF2BP2 accumulates and recruits the exonuclease XRN2, thereby promoting the degradation of the RNA transcript that encodes ATP6V1A, a major catalytic subunit of the v-ATPase.

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Fig. 3. IGF2BP2 is a SIRT1 substrate. (A) Representative immunoblots of WCLs, as well as immunoprecipitations using an acetylated lysine antibody (acetyl K-IP) performed on the WCLs, from control (CTRL) and SIRT1 KD cells probed for IGF2BP2, SIRT1, p53, tubulin, and β-actin as a loading control. (B) Mass spectrometry profiling showing the acetylated peptide identified in FLAG-tagged IGF2BP2, referred to in this figure as its alternative name IMP2, was immunoprecipitated from SIRT1 KD cells ectopically expressing this construct. The sequence, shaded in blue, represents the identified peptide and the location of the acetylated lysine (lysine 530) is highlighted (red). (C) Representative immunoblots of WCLs, as well as immunoprecipitations using a FLAG antibody (FLAG IP) performed on the WCLs, from control and SIRT1 KD MDA-MB-231 cells ectopically expressing the indicated FLAG-tagged IGF2BP2 proteins probed for acetylated lysine residues (Acetyl K), SIRT1, the FLAG-tagged proteins, and CNOT1 as a loading control. (D) Representative immunoblots of WCLs, as well as immunoprecipitations using a FLAG antibody (FLAG IP) performed on the WCLs, from control and SIRT1 KD MDA-MB-231 cells ectopically expressing FLAG-tagged IGF2BP2 WT or the K530R mutant. The immunoprecipitated proteins were treated without (−) or with a purified recombinant form of SIRT1, and then probed for acetylated lysine residues (Acetyl K). (E) Immunoblots of WCLs from control (CTRL) and SIRT1 KD MDA-MB-231 cells ectopically expressing either FLAG-tagged IGF2BP2 WT or the K530R mutant probed for SIRT1, ATP6V1A, the FLAG-tagged proteins, and β-actin as a loading control. (F) Immunoblots of WCLs from MDA-MB-231 cells ectopically expressing the indicated forms of FLAG-tagged IGF2BP2 probed for the FLAG-tagged proteins, SIRT1, and β-actin as a loading control. RT-qPCR was performed on the immunoprecipitated FLAG-tagged proteins to determine the levels of the ATP6V1A transcript that associated with each construct (Top). The relative expression levels of the indicated proteins, relative to controls, in A, C, D, and E were quantified based on densitometry and shown above the bands. The data shown in F is presented as mean ± SD. Statistical significance was determined using a one-way ANOVA test; ns, not significant.
**Fig. 4.** XRN2 associates with the acetylated form of IGF2BP2 and promotes ATP6V1A transcript degradation. (A) Table showing proteins that regulate RNA stability and were found to interact with IGF2BP2 by mass spectrometry. The numbers of peptide spectrum matches (# PSMs) identified for each protein are listed. (B) Representative immunoblots of WCLs, as well as immunoprecipitations using a FLAG antibody (FLAG IP) performed on the WCLs, from control and SIRT1 KD cells ectopically expressing FLAG-tagged IGF2BP2 and probed for XRN2, YBX1, the FLAG-tagged construct, and SIRT1. (C) Representative immunoblots of WCLs, as well as immunoprecipitations using a FLAG antibody (FLAG IP) performed on the WCLs, from control and SIRT1 KD cells ectopically expressing the indicated FLAG-tagged forms of IGF2BP2 probed for XRN2, the FLAG-tagged proteins, and SIRT1. (D) Quantification of the relative amount of XRN2 that immunoprecipitated with each of the FLAG-tagged IGF2BP2 constructs shown in C. (E) RT-qPCR was performed on SIRT1 KD cells (control; CTRL), or on SIRT1/CNOT1, SIRT1/XRN2, or SIRT1/IGF2BP2 double-KD cells, to determine the relative expression levels of the ATP6V1A transcript (relative to actin transcript levels) (Top). Representative immunoblots of the cells described above (WCLs) probed for SIRT1, CNOT1, XRN2, IGF2BP2, and β-actin as a loading control (Bottom). (F) Quantification of the NTA shown in SI Appendix, Fig. S5C performed on the conditioned media collected from an equivalent number of SIRT1 KD, and SIRT1/XRN2 double-KD cells. (G) Representative immunoblots of exosome lysates (EXO) and WCLs prepared from control (+), SIRT1 KD, and SIRT1/XRN2 double-KD cells probed for ubiquitinated proteins, SIRT1, XRN2, and HSP90 and β-actin as loading controls. The relative expression levels of the indicated proteins, relative to controls, in B and G were quantified based on densitometry and shown above the bands. The data shown in D, E, and F represent means ± SD. Statistical significance in D, E, and F was determined using a one-way ANOVA test: ****P < 0.0001, ***P < 0.001 **P < 0.01, *P < 0.05; ns, not significant.
Materials and Methods

SI Appendix contains all of the relevant information regarding the experimental models and subject details used in this study, including all cell lines and our tumor mouse models. It also contains detailed descriptions of all approaches performed and information regarding the reagents used in each of them, as well as lists of antibodies used for immunoblot analysis and experiments involving immunoprecipitations, primer sets used to generate the indicated expression constructs and for performing qRT-PCR, and sequences of the shRNAs.

Data, Materials, and Software Availability. All study data are included in the article and/or SI Appendix.

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