IMP3 RNP Safe Houses Prevent miRNA-Directed HMGA2 mRNA Decay in Cancer and Development

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SUMMARY

The IMP3 RNA-binding protein is associated with metastasis and poor outcome in human cancer. Using solid cancer transcriptome data, we found that IMP3 correlates with HMGA2 mRNA expression. Cytoplasmic IMP3 granules contain HMGA2, and IMP3 dose-dependently increases HMGA2 mRNA. HMGA2 is regulated by let-7, and let-7 antagomiRs make HMGA2 refractory to IMP3. Removal of let-7 target sites eliminates IMP3-dependent stabilization, and IMP3-containing bodies are depleted of Ago1-4 and miRNAs. The relationship between Hmga2 mRNA and IMPs also exists in the developing limb bud, where IMP1-deficient embryos show dose-dependent Hmga2 mRNA downregulation. Finally, IMP3 ribonucleoproteins (RNPs) contain other let-7 target mRNAs, including LIN28B, and a global gene set enrichment analysis demonstrates that miRNA-regulated transcripts in general are upregulated following IMP3 induction. We conclude that IMP3 RNPs may function as cytoplasmic safe houses and prevent miRNA-directed mRNA decay of oncogenes during tumor progression.

INTRODUCTION

In principle, all posttranscriptional steps can have profound effects on the expression of onco- and tumor-suppressor genes and factors that maintain genome integrity. Although more than 500 different RNA-binding proteins participate in mRNA events, only a handful of these proteins have been directly implicated in neoplasia. Insulin-like growth factor 2 (IGF2) mRNA-binding protein 3 (IMP3, IGF2BP3) belongs to a family of mRNA-binding proteins (IMP1, IMP2, and IMP3) that are involved in RNA localization, translation, and stability (Christiansen et al., 2009). IMP1 and IMP3 form heterodimers and exhibit similar RNA-binding specificities (Nielsen et al., 2004). They are almost exclusively produced during embryogenesis with early maternal and zygotic expression (for review, see Nielsen et al., 2001), followed by a peak at mouse embryonic days 10.5–12.5 (E10.5–E12.5) (Nielsen et al., 1999; Runge et al., 2000). IMPs are mainly cytoplasmic and form large motile ribonucleoprotein (RNP) granules dispersed around the nucleus and in cellular protrusions (Nielsen et al., 2002). These RNP granules, or “locasomes,” are a unique RNP entity distinct from neuronal hstauen and/or fragile X mental retardation protein granules, processing bodies (P bodies), and stress granules (Jønson et al., 2007). Because granules contain CBP80 and factors belonging to the exon-junction complex, and lack eIF4E, eIF4G, and 60S ribosomal subunits, it is conceivable that mRNAs in these bodies have never been translated.

IMP3 is found in a variety of cancers. IMP3-positive tumors are prone to metastatic behavior and poor outcome (Beljan Perak et al., 2012; Chen et al., 2011, 2013; Hoffmann et al., 2008; Jeng et al., 2008; Ji et al., 2008b; Kim et al., 2012; Købel et al., 2009; Li et al., 2011; Lochhead et al., 2012; Lu et al., 2011; Mentrikoski et al., 2009; Schaeffer et al., 2010; Sthekova et al., 2008; Szarvas et al., 2012; Yantiss et al., 2008), and IMP3 is widely used to depict metastatic tumor cells during staging (Kapoor, 2008; Kim and Cha, 2011; Mentrikoski et al., 2009; Mhawech-Fauceglia et al., 2010; Yantiss et al., 2008). In support of a causal role in transformation, transgenic overexpression of mouse IMP3/KOC leads to increased cell proliferation and metaplasia of pancreatic acinar cells (Wagner et al., 2003), and in mammary-cancer-prone mice, overexpression of IMP1 causes metastatic cancer (Tessier et al., 2004). Although there is clear support for a direct role of IMP3 in tumorigenesis, the mechanisms by which IMP3 elicits its effects are incompletely understood.

To unravel the mechanism by which IMP3 promotes tumor growth, we employed global transcriptome data from a large set of solid cancers, and observed that IMP3 was correlated with oncogenic HMGA2 mRNA expression. Similarly to IMP3, HMGA2 is an oncofetal protein that is primarily expressed during embryogenesis (Chiappetta et al., 1996; Zhou et al., 1995). HMGA2 is tumorigenic and associated with poor prognosis and low overall survival (reviewed in Fusco and Fedele, 2007). The HMGA2 3’ UTR has considerable potential for posttranscriptional regulation by RNA-binding proteins and miRNA-induced silencing complexes (miRISCs) with its seven let-7 miRNA target sites in HMGA2 3’ UTR (Lee and Dutta, 2007; Mayr et al., 2007).
We show that IMP3 RNP granules contain large amounts of HMGA2 transcripts, and that IMP3 locasomes protect and upregulate HMGA2 by opposing the intersection between Ago2/let-7 and HMGA2 mRNA. IMP3 RNP granules also contain a number of other let-7 target mRNAs, including the pluripotency factor LIN28B mRNA, that promote tumor growth and embryonal development by inhibiting let-7 biogenesis. A global gene set enrichment analysis (GSEA) analysis demonstrated that the protective role of IMP3 includes miRNA-regulated transcripts in general. We propose that IMP3 locasomes may function as cytoplasmic safe houses that prevent miRNA-directed mRNA decay of proto-oncogenes during tumor progression and embryogenesis.

RESULTS

IMP3 and HMGA2 mRNA Are Coexpressed in Human Cancers

High levels of RNA-binding IMP3 have been associated with poor outcome and metastasis in cancers of the lung, kidney, colorectal, breast, and ovary (Beljan Perak et al., 2012; Bellezza et al., 2009; Findeis-Hosey et al., 2010; Hoffmann et al., 2008; Jiang et al., 2008a, 2008b; Köbel et al., 2009; Lochhead et al., 2012; Walter et al., 2009; Yuan et al., 2009). To identify putative oncogenic mechanisms connected to IMP3, we examined IMP3 mRNA expression in a series of 270 tumors from the above cancers and searched for transcripts that were correlated with the presence of IMP3.

IMP3 was expressed at high levels in ~45% of the tumors. Compared with background in the negative tumors, IMP3 levels were on average ~6.5-fold higher among positive tumors. Some positive tumors exhibited an up to 40-fold increase in IMP3 (Figure 1A). To identify transcripts that were associated with IMP3 expression, we first employed a two-way comparison of IMP3-positive tumors (>6 IMP3 log2 values) and IMP3-negative tumors (<4.5 IMP3 log2 values) to depict enriched transcripts (Figure 1B). We found that 163 transcripts were enriched in IMP3 tumors after setting a p value cutoff at 1E-10 and a q value of 1E-10. The most enriched transcript encoded HMGA2 (p = 1.9 × 10⁻¹³), followed by ARNTL2, TOP2A, IGF2BP2 (IMP2), SLC2A1, and MTHFD1L. The enrichment of IMP2 is in agreement with the fact that HMGA2 promotes transcription of the IMP2 gene (Brants et al., 2004; Li et al., 2012). A GSEA in the molecular signatures database MSigDB (http://www.broadinstitute.org) showed that IMP3-associated mRNAs overlapped with the Gene Ontology (GO) terms Cell Cycle_Process (p = 6 × 10⁻¹⁵), Mitotic_Cell_Cycle (p = 8 × 10⁻¹⁵), Spindle (p = 1 × 10⁻¹⁰), and Cell Cycle_Phase (p = 6 × 10⁻¹⁰), indicating that IMP3-positive tumors, in agreement with previous reports, were characterized by fast proliferation (Hartmann et al., 2012). We subsequently examined a larger collection of 899 cancers from the above groups and showed that the Pearson correlation coefficient between IMP3 and HMGA2 among all groups was 0.44 with a false discovery rate (FDR) q value of 2.7 × 10⁻⁵, ranging from 0.12 in breast cancers to 0.5 in lung cancers (Figure 1C). A closer analysis of the breast cancer group showed that the lower correlation in these tumors could be explained by the selective expression of IMP3 in triple-negative breast cancers, whereas HMGA2 is also found in other histological classes of breast tumors.

To substantiate the causal relation between IMP3 and HMGA2, we generated a line of HT1080 cells expressing 3xFLAG-tagged IMP3 under control of a tetracycline-inducible promoter, and immunoprecipitated IMP3 RNP after tetracycline
induction. Coimmunoprecipitated mRNAs were identified by deep sequencing, and total RNA was also sequenced to determine the relative enrichment of transcripts in IMP3 RNP. A total of 850 transcripts were enriched >3-fold compared with the number in the initial cell-lysate (CLS +tet) versus CL(+tet). Reads, coverage, and number of transcripts are also indicated. The putative let-7 target sites were identified by submitting the gene symbols to the miRanda or Targetscan databases. The SVR scores of the individual elements are indicated.

**IMP3 Upregulates HMGA2 mRNA and Protein**

To further substantiate the causal relation between IMP3 and HMGA2, we employed an additional stable HT1080 cell line expressing FLAG-tagged HMGA2 protein. Whereas overexpression of FLAG-HMGA2 protein had no effect on the IMP3 level (data not shown), induction of 3xFLAG-IMP3 (Figure 2A) was followed by a time-dependent rise in the steady-state level of endogenous 4.5 kb HMGA2 mRNA by a factor of 2.5 after 12 hr (Figure 2B). Similar results were obtained with 3xFLAG-IMP3 (Figure 2C). There was no indication of alternative HMGA2 mRNA splicing or poly(A) site usage. Figure 2C summarizes the quantification of the data from the western and northern blot analyses.

To examine whether the increased steady-state level of HMGA2 mRNA was followed by an increased amount of HMGA2 protein, we performed a western blot analysis in the absence or presence of tetracycline (Figure 2D). Corresponding to the increase in HMGA2 mRNA, HMGA2 protein levels increased 2.1-fold. Mixing uninuced and tetracycline-induced cells followed by immunocytochemical staining of either nuclear HMGA2 protein (green color) or cytoplasmic 3xFLAG-IMP3 (red color, Figures 2E and 2F) showed that nuclear HMGA2 staining, quantified by confocal microscopy, also was ~2-fold higher in tetracycline-induced cells.

Finally, we determined the translational status of HMGA2 mRNA following IMP3 induction by means of polysome isolation (Figures 3A and 3B). The analysis provided no indications of altered translational initiation in the presence of IMP3, since HMGA2 4.5 kb mRNA sedimented in polysomal fractions 4–8 regardless of tetracycline addition. 3xFLAG-IMP3 was mainly associated with IMP3 RNP (Table 1). Taken together, these results demonstrate that IMP3 and HMGA2 mRNAs are strongly correlated in solid cancers. Moreover, HMGA2 mRNA is physically associated with IMP3 RNP in vivo.

**Table 1. List of the 20 Most Upregulated Transcripts after Induction of 3x-FLAG-IMP3**

| mRNA | Reads | Median Coverage | RPKM | FC +tet versus – tet | FC IP(+tet) versus CL(+tet) | No. of Transcripts | Let-7 SVR Scores | let-7 Targets
<table>
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<tr>
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<tr>
<td>IMP3</td>
<td>2,729</td>
<td>34</td>
<td>20.9</td>
<td>16.1</td>
<td>–1.1</td>
<td>–</td>
<td>(–1.1418)</td>
<td>√ (8-mer)</td>
</tr>
<tr>
<td>HMGA2</td>
<td>14,139</td>
<td>164</td>
<td>81.6</td>
<td>2.4</td>
<td>8.9</td>
<td>1,720.0</td>
<td>(–0.3281, –0.9940, –0.2490, –0.8742, –0.1048)</td>
<td>√ (8-mer)</td>
</tr>
<tr>
<td>IL1B</td>
<td>793</td>
<td>42</td>
<td>18.0</td>
<td>2.1</td>
<td>2.0</td>
<td>75.2</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>ANKRD46</td>
<td>377</td>
<td>6</td>
<td>3.1</td>
<td>2.1</td>
<td>–1.2</td>
<td>–</td>
<td>(–0.2436)</td>
<td>√ (7-mer-m8)</td>
</tr>
<tr>
<td>BACH1</td>
<td>845</td>
<td>26</td>
<td>11.1</td>
<td>2.0</td>
<td>4.0</td>
<td>88.3</td>
<td>(–0.5106, –1.0348)</td>
<td>√ (8-mer)</td>
</tr>
<tr>
<td>THBS1</td>
<td>11,330</td>
<td>151</td>
<td>68.4</td>
<td>1.9</td>
<td>–1.2</td>
<td>–</td>
<td>(–0.1594)</td>
<td>√ (7-mer)</td>
</tr>
<tr>
<td>S1PR1</td>
<td>597</td>
<td>16</td>
<td>7.0</td>
<td>1.8</td>
<td>2.7</td>
<td>34.9</td>
<td>none</td>
<td></td>
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<td>ZCCHC3</td>
<td>660</td>
<td>17</td>
<td>8.4</td>
<td>1.8</td>
<td>–1.7</td>
<td>–</td>
<td>(–0.9080)</td>
<td>√ (7-mer-m8)</td>
</tr>
<tr>
<td>ITGB8</td>
<td>867</td>
<td>7</td>
<td>3.2</td>
<td>1.8</td>
<td>2.2</td>
<td>12.5</td>
<td>(–0.1705)</td>
<td>√ (7-mer-m8)</td>
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<td>ARL5B</td>
<td>480</td>
<td>9</td>
<td>4.6</td>
<td>1.8</td>
<td>4.1</td>
<td>33.5</td>
<td>none</td>
<td></td>
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<tr>
<td>ANKRD1</td>
<td>1,716</td>
<td>69</td>
<td>29.2</td>
<td>1.8</td>
<td>–3.0</td>
<td>–</td>
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<td>FLAG1</td>
<td>447</td>
<td>4</td>
<td>2.0</td>
<td>1.8</td>
<td>4.7</td>
<td>16.2</td>
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<td>SEMA3A</td>
<td>432</td>
<td>4</td>
<td>2.3</td>
<td>1.7</td>
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<td>14.3</td>
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<td>CPA4</td>
<td>2,491</td>
<td>62</td>
<td>29.0</td>
<td>1.7</td>
<td>2.0</td>
<td>95.7</td>
<td>(–0.9174)</td>
<td>√ (8-mer)</td>
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<td>TMG2</td>
<td>10,364</td>
<td>193</td>
<td>87.4</td>
<td>1.7</td>
<td>1.4</td>
<td>204.5</td>
<td>none</td>
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<tr>
<td>TNFRSF11B</td>
<td>332</td>
<td>11</td>
<td>4.5</td>
<td>1.7</td>
<td>1.4</td>
<td>10.6</td>
<td>none</td>
<td></td>
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<tr>
<td>LIN28B</td>
<td>334</td>
<td>4</td>
<td>1.9</td>
<td>1.7</td>
<td>2.5</td>
<td>8.1</td>
<td>(–0.3931, –0.3511, –0.2389, –0.1497)</td>
<td>√ (8-mer)</td>
</tr>
<tr>
<td>IRS2</td>
<td>865</td>
<td>13</td>
<td>7.1</td>
<td>1.6</td>
<td>–1.3</td>
<td>–</td>
<td>(–0.3305)</td>
<td>√ (8-mer)</td>
</tr>
<tr>
<td>I1RAP</td>
<td>650</td>
<td>7</td>
<td>3.5</td>
<td>1.6</td>
<td>1.6</td>
<td>9.0</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>C9orf6</td>
<td>793</td>
<td>33</td>
<td>14.3</td>
<td>1.6</td>
<td>9.0</td>
<td>208.2</td>
<td>none</td>
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Cultured cells were stimulated with vehicle or tetracycline for 24 hr before total RNA or IMP3 locasomes were isolated. Total RNA and transcripts associated with the IMP3 RNP were subjected to RNA sequencing. The results represent three independent experiments. The table includes the fold change after tetracycline-induction (FC +tet versus –tet) and the fraction of immunoprecipitated reads compared with the number in the initial cell-lysate (FC IP(+tet) versus CL(+tet)). Reads, coverage, and number of transcripts are also indicated. The putative let-7 target sites were identified by submitting the gene symbols to the miRanda or Targetscan databases. The SVR scores of the individual elements are indicated.
Consequently, we considered whether IMP3 would oppose the actions of let-7 miRNA on HMGA2 mRNA. Segmentation of HMGA2 mRNA and UV crosslinking with recombinant IMP3 or lysates from uninduced or tetracycline-induced TREPX1080 cells initially showed that IMP3 exhibited strong binding to the HMGA2 3’ UTR (Figure S2). Endogenous let-7 isoforms in HT1080 cells were inhibited by a locked nucleic acid (LNA) antagomiR directed toward the let-7 family in the absence or presence of tetracycline (Figure 4A). A mock transfection or transfection with the unrelated antagomiR anti-miR-449b gave similar results in terms of elevated HMGA2 mRNA expression following 3xFLAG-IMP3 induction. In
and 6&7 exhibited increased firefly activity upon 3xFLAG-IMP3 induction. We observed a nonadditive effect of 3xFLAG-IMP3 induction both on endogenous HMGA2 mRNA employing a let-7 antagomiR and on a luciferase reporter containing mutated let-7 target sites, implying that IMP3 protects HMGA2 mRNA from the RISC pathway.

To corroborate the functional significance of IMP3-dependent let-7 interference, we examined the proliferative capacity of 3xFLAG-IMP3 in the absence or presence of the let-7 family antagomiR. As shown in Figure 4D, the number of cells that overexpressed IMP3 began to increase 10–12 hr following the addition of tetracycline. This coincided with the increase of the HMGA2 transcript, which was visible after 6 hr of tetracycline treatment and continued to increase for an additional 10 hr (Figures 2B and 2C). The effect of 3xFLAG-IMP3 expression on the doubling time was similar to that observed after addition of the let-7 family antagomiR, and the two treatments were not additive. The reduction in doubling time upon 3xFLAG-IMP3 addition is 13%, which will generate a 74-fold difference in tumor volume after 1 month (720 hr). Knockdown of HMGA2 mRNA was followed by a clear inhibition of cell proliferation and eliminated the effect of IMP3 (Figure 4E).

**IMP3 Locasomes Provide a RISC-Free Cytoplasmic Repository for HMGA2 mRNA**

Because the luciferase reporter assay of HMGA2 3’ UTR segment 2&3 in Figures 4B and 4C showed that downregulation of luciferase expression by let-7 miRNA could be partially rescued by IMP3, we examined the possibility of a direct competition between let-7g miRNA and IMP3 for the HMGA2 segment 2&3 (3’ UTR positions 1,088–1,306). The electrophoretic mobility-shift analysis shown in Figure 5A indicates that recombinant IMP3 is able to associate with radiolabeled segment 2&3 with a Kd of ~1 nM, and the analysis in Figure 5B reveals that radiolabeled let-7g miRNA is able to associate with its target RNA in segment 2&3, regardless of the presence of IMP3. To pinpoint the interactions between the three species at the nucleotide level, we carried out a selective 2’-hydroxyl acylation analyzed by primer extension (SHAPE) analysis of segment 2&3 RNA (McGinnis et al., 2012). Figure 5C shows that in the presence of IMP3 alone, acylations are enhanced in the seed region and its target, regardless of the presence of let-7g. In contrast, outside the seed target region in segment 2&3, the acylation behavior is dictated by IMP3 rather than let-7g miRNA. We infer that IMP3 and let-7g miRNA are able to associate simultaneously with HMGA2 segment 2&3 in vitro, so the opposing effect observed in vivo is unlikely to result from a straightforward competition between let-7g miRNA and IMP3 for the HMGA2 3’ UTR seed target.

Because upregulation of HMGA2 mRNA was unlikely to result from a direct sterical hindrance of let-7 miRNA binding, we examined whether IMP3 RNP represents a RISC-free environment. Immunoprecipitations of cytoplasmic lysates in the presence of 0.5 mM EDTA with Ago2 or FLAG antibodies (Figure 6A) revealed that endogenous full-length HMGA2 mRNA was virtually undetectable in the anti-Ago2 immunoprecipitate. The
Figure 4. IMP3-Induced Increase of HMGA2 mRNA Depends on let-7 miRNA

(A) Endogenous HMGA2 mRNA levels after 3xFLAG-IMP3 induction in the presence of mock, anti-miR-449b, or anti-let-7 LNA antagomiR. The LNA miCurry inhibitors were transfected 16 hr before the cells were split and cultured in media with or without tetracycline. The data represent three independent experiments and are presented as mean ± SD.

(B) Schematic representation of full-length HMGA2 mRNA and the firefly luciferase reporter constructs encompassing two let-7 target sites 2&3 (green) and 6&7 (blue) examined in the absence or presence of 3xFLAG-IMP3. Colored boxes depict reading frames, UTRs are depicted as gray lines, and 2&3 and 6&7 designate RNA segments that exhibit strong IMP3 binding. In addition to the two wild-type segments inserted downstream of firefly luciferase, two other constructs were made in which two nucleotides in the seed target sequence (UACCUC→UGCGUC) were mutated.

(C) Activation of the luciferase reporters outlined in (B) by 3xFLAG-IMP3 in the presence of intact or mutated let-7 target sequences. The y axis represents the relative firefly luciferase activity divided by the Renilla luciferase activity. In the segment 2&3 construct, both let-7 target sites were mutated (HMGA2 Seg 2 m&3 m), whereas only the biologically active target site was mutated in the segment 6&7 construct (HMGA2 Seg 6 m&7). The data originate from three independent experiments and are presented as the mean ± SD.

(legend continued on next page)
rRNA content was performed after siRNA transfection and visualized by gel electrophoresis. The data represent three independent measurements and are presented as mean ± SD. Quantitative PCR of the cellular HMGA2 mRNA and 18S rRNA content was performed after siRNA transfection and visualized by gel electrophoresis.

Let-7 Targets in IMP3 RNP Granules

IMP3 locasomes have been proposed to represent posttranscriptional regulons (Jønson et al., 2011). To generalize our observations concerning IMP3 and miRNA activity, and reveal additional transcripts that could play a role in cancer progression, we compared global RNA sequencing profiles from cells treated with and without tetracycline with the transcripts identified in the particles. Among the 20 most upregulated transcripts, eight of 19 (excluding IMP3) exhibited let-7 seed target sequences according to the miRanda database, and nine of 19 exhibited them when we employed Targetscan to predict the sequences according to the miRanda database, and nine of 19 exhibited them when we employed Targetscan to predict the sequences. As shown in Table 1, the HMGA2 transcript was the most upregulated transcript (2.4-fold) upon IMP3 induction, followed by ANKR46, BACH1, THBS1, ZCCHC3, ITGB8, CPA4, IRIS2, and LIN28B. HMGA2 was strongly enriched (9-fold) in the particles together with let-7 target-containing BACH1, ITGB8, CPA4, and LIN28B. A number of the remaining enriched transcripts were targets for other miRNAs, in particular miR-181a/b (data not shown).

RNA-binding LIN28B blocks the nuclear conversion of the primary let-7 transcript to pre-let-7 (Heo et al., 2008; Viswanathan et al., 2008), and LIN28B promotes malignant growth by opposing let-7 (Viswanathan et al., 2009). Moreover, LIN28B is expressed at the same developmental stages as IMPs (Yang and Moss, 2003) and controls body size similarly to HMGA2 and IMP1 (Shinoda et al., 2013). Therefore, we considered whether IMP3 locasomes represent a regulon that integrates the expression of LIN28B and HMGA2 by opposing let-7. We
examined the effect of IMP3 and let-7 antagomiRs on LIN28B mRNA expression (Figure S5), and found that similarly to HMGA2 mRNA, LIN28B mRNA was induced ~2-fold after 3xFLAG-IMP3 induction. Let-7 antagomiRs increased the steady-state level of LIN28B mRNA 3-fold, and it was not possible to impose a further increase of the LIN28B transcript following addition of 3xFLAG-IMP3. MiR-449, which was included as a control, had no effect on the LIN28B mRNA level. For comparison, we also examined the effects of 3xFLAG-IMP3 and let-7 antagomiRs on IMP1 expression. IMP1 mRNA exhibits let-7 target sites in the 3’ UTR (Boyerinas et al., 2008), but in contrast to HMGA2 and LIN28B mRNAs, the transcript is not associated with IMP3 RNP. In agreement with a protective activity of granule association, IMP1 mRNA was not increased upon induction of 3xFLAG-IMP3, whereas addition of let-7 antagomiRs increased the level of IMP1 transcripts.

To examine whether IMP granules exhibit a more generalized protection of miRNA-targeted mRNAs, we generated a list of miRNA-regulated mRNAs by combining Targetscan predictions with the expression of miRNAs and mRNAs as described
previously (Gallagher et al., 2010). To validate the list of putative miRNA targets, we first performed a GSEA with the top 1,000 transcripts that were predicted to be most repressed in HEK293 cells in a previous Ago2 knockdown analysis from Schmitter et al (2006) (Figure S6A). This showed that the predicted miRNA-regulated transcripts were clearly enriched following Ago2 knockdown.

To study whether IMP1 or IMP3 imposed a global protection against miRNAs, we performed GSEAs using the top 300 miRNA-regulated mRNAs in the two-group comparisons of tetracycline-induced 3xFLAG-IMP1 HT1080 cells (Figure S6B), 3xFLAG-IMP3 HT1080 cells (Figure S6C), and 3xFLAG-IMP1 HEK293 cells (Figure S6D) versus noninduced cells. In all cases, we observed a significant ($p < 0.001$) enrichment of the miRNA-repressed mRNAs following IMP overexpression. As expected, not every miRNA-targeted transcript became upregulated, conceivably because IMPs bind different mRNAs with varying affinity. We therefore examined whether transcripts that were highly embedded in the granules would be insensitive to Ago2 knockdown compared with transcripts in which only a small fraction of the total pool was bound to IMP granules. The analysis showed that tightly associated mRNAs were unresponsive to Ago2 knockdown (NES = $-0.92$, $p = 0.549$) (Figure S6F), whereas less-granule-associated mRNAs were enriched following Ago2 depletion (NES = 1.42, $p = 0.002$) (Figure S6E), in agreement with a model in which granule-associated mRNAs, in general, are less accessible to the RISC complex. Taken together, these results indicate that IMP1 and IMP3 granules provide a generalized protection against miRNA-mediated mRNA decay.

**Interplay among Hmga2 mRNA, let-7 miRNA, and IMP1 during Limb Bud Development**

To establish whether the effects observed in HT1080 cells also occurred in the intact organism, we directed our attention toward the developing mouse. Figure 7A is a schematic outline of the reciprocal expression of Hmga2 mRNA and let-7 miRNA during mouse development (Park et al., 2007) upon which the narrow temporal expression of the IMP family has been superimposed (Nielsen et al., 1999). Characteristically, IMPs are present at the stage where there is a sharp posttranscriptional downregulation of Hmga2 mRNA mediated by increasing amounts of let-7 mRNA. Therefore, it ought to be feasible for IMPs to adjust the expression of Hmga2, provided a similar spatial expression pattern is observed.

IMP1 and IMP3 exhibited similar binding to Hmga2 segment 2&3 and segment 6&7 (Figure 7B), and, as described above, both factors induced Hmga2 to the same extent (Figure S1). We employed Imp1 mice because IMP1 is the dominant fetal isoform, whereas IMP3 is predominantly expressed during carcinogenesis. In situ hybridizations with either an Imp1 probe (Figure 7C, pictures 1 and 2) or an Hmga2 probe (Figure 7C, pictures 3 and 4) at E12.5 showed overlapping staining with both probes in the limb bud. Therefore, limb buds from wild-type Imp1, heterozygous Imp1/−, and knockout Imp−/− mice were examined at E13.5 and E14.5 to achieve different concentrations of IMP1 protein. In parallel, quantification of a northern blot analysis of full-length Hmga2 mRNA revealed that there was a dose-depen-
dent decrease in the Hmga2 mRNA level upon IMP1 depletion (Figures 7D and 7E). We infer that the interplay between Hmga2 mRNA and the IMP RNA-binding protein family, as seen in a human cancer cell line, is also present in the developing limb bud in the mouse.

**DISCUSSION**

To search for molecular targets of IMP3, we employed a large number of genome-wide expression profiles from different solid tumors and searched for mRNAs that were correlated with IMP3 expression. This approach is appealing because it requires no a priori assumption of possible interactions. Moreover, the data reflect the situation in real solid tumors. We discovered a positive association between Hmga2 and IMP3, which we examined further because Hmga2 is a well-established oncogene and overlaps temporally and spatially with IMP3 during embryogenesis (Cleynen and Van de Ven, 2008).

IMP3 was physically associated with and upregulated Hmga2 mRNA and protein, with no apparent effect on translation initiation. Since IMP3 resides in stable cytoplasmic RNP granules, it was conceivable that IMP3 stabilized Hmga2 mRNA. This let us to examine the effect of IMP3 on let-7-dependent downregulation of Hmga2 mRNA. Although RNA-binding proteins such as IMPs may affect miRNA binding (Elcheva et al., 2009) and miRNA biogenesis, or alter the secondary structure of miRNA target sites (reviewed in van Kouwenhove et al., 2011), we were unable to reveal a straightforward competition between IMP3 and let-7 for a common cis element. Instead, our results point toward an additional possibility that has implications for our understanding of mRNA locasomes/granules. Both the immunoprecipitation and colocalization experiments showed little, if any, overlap between IMP3 and Ago2, which is compatible with distinct cytoplasmic domains for IMP3 and Ago2. In this way, IMP3 locasomes may be regarded as safe houses for mRNAs, where they are protected from degradation on the way to their final destinations and translation. As mentioned above, transcripts have not completed their pioneering round of translation, so it is appealing to regard locasomes as an extension of the nuclear compartment in the cytoplasm.

The physical details of IMP granules, and RNP granules in general, are beginning to emerge. Recent studies of synaptic RNP granules have shown that they exit the nucleus by budding through the inner and outer membranes via a nuclear egress mechanism (Speese et al., 2012). Aggregation and recruitment into condensed hydrogels are likely to occur through polymerization of low-complexity sequences present in RNA-binding proteins (Han et al., 2012; Kato et al., 2012). In agreement with these observations, IMP granules are dynamic and exhibit a diameter in the range of 100–300 nm. Particles may contain several hundred protein molecules roughly the size of IMPs and 10–30 mRNA transcripts (Jønson et al., 2007), and their “nuclear” status is supported by the presence of PABN1/PABP-2 (Speese et al., 2012). It remains to be resolved how Ago2/RISC is excluded from the locasomes, but a combination of steric hindrance by the dense protein core and a lack of low-complexity sequences that are required for sustained association with the particles (Weber and Brangwynne, 2012) may play a role.
Several transcripts in the locasomes contained let-7 target sites (Dogar et al., 2014; Hu et al., 2013; Yun et al., 2011; Zhu et al., 2011) and became upregulated upon IMP3 induction. One of these was LIN28B mRNA, which, similarly to IMP3 and HMGA2, has been implicated in various developmental processes and cancer (reviewed by (Thornton and Gregory, 2012). LIN28B blocks the production of mature let-7 miRNA (Heo et al., 2008; Viswanathan et al., 2008), and this promotes malignant growth (Molenar et al., 2012; Viswanathan et al., 2009). In humans and mice, IMP3/IMP1, HMGA2, and LIN28B are involved in growth and differentiation, and the deletion of either factor in mice leads to dwarfism (Hansen et al., 2004; Shinoda et al., 2013; Zhou et al., 1995). The physiological significance of the intersection is illustrated by the lowered Hmga2 mRNA levels in the E13.5 limb buds of our Imp1 knockout mice, which emphasizes the critical importance of RNA-binding protein concentration in the intact organism. LIN28B mRNA is coexpressed with members of the IMP family during midgestation, particularly in epithelia (Yang and Moss, 2003), and LIN28B mRNA is also coexpressed with IMP3 mRNA in a number of cancers (data not shown). Therefore, taken together, our findings indicate that the activity of HMGA2, LIN28B, and let-7 is coordinated by heterochronic IMP3 during embryogenesis and carcinogenesis.
Taken together, the IMP3-mediated alleviation of let-7 activity toward HMGA2 and LIN28B mRNAs may explain why poor outcome is a hallmark of IMP3-positive tumors. Moreover, our data demonstrate how posttranscriptional mechanisms may be deeply involved in tumor progression, and we propose that segregation of transcripts into IMP3 RNPs may be a general mechanism that prevents miRNA-dependent mRNA decay during tumor growth.

**EXPERIMENTAL PROCEDURES**

**Tumor Gene-Expression Profiles**

Expression profiles of tumor samples were downloaded from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) or generated from samples collected and processed at our own facility at Rigshospitalet. All tumors were analyzed on human U133 Plus 2.0 arrays (Affymetrix). Cell files were preprocessed using the robust multichip average (RMA) method (Bolstad et al., 2003) and evaluated for quality parameters using the Simpleaffy functionality of the R/Bioconductor packages. The data were visualized and analyzed using the Quicore Omics Explorer software, and the probe sets employed in the two-way comparison and Pearson correlation coefficient were IMP3 (203820_s_at) and HMGA2 (208025_s_at).

**Inhibition of let-7 Family Members**

A mixture of LNA antagonimicros was produced to specifically inhibit all members of the let-7 family of miRNAs. The mixture consisted of five custom-designed LNA-containing oligonucleotides (hsa-let-7a-3d CAACCTACTACCTC, hsa-let-7e ACAACTCTACTCTC, hsa-let-7i ACAATCTACTCTTC, hsa-let-7g-1 ACAAAACTACTCTCA, and hsa-miR-98 CAACATTACTCCTCA). Upon transfection of the let-7 family inhibitor mixture (50 nM) into TREX1080 3xFLAG-IMP3 cells, tetracycline was added to induce transcription of the 3xFLAG-IMP3 construct. As control, an LNA inhibitor against miR-449b was used (CCAGCTAACAATACACTGCCT). Transfections were performed with Turbofect (Fermentas). Cells were harvested 24 hr after transfection and subsequently lysed in TritZol (Invitrogen).

**Isolation of 3xFLAG-IMP3 and Ago2 RNPs**

Anti-Ago2 agarose beads were prepared with protein A/G, and immunoprecipitation of 3xFLAG-IMP3 was performed as previously described using anti-FLAG agarose beads from Sigma-Aldrich (Janson et al., 2011).

**SHAPE Probing**

For SHAPE probing, 10 nM of HMGA2 3’ UTR segment 1,088–1,306 was reanimated in 20 mM Tris-HCl, pH 7.5, 140 mM KCl, 2 mM MgCl2, and 0.1% Triton X-100 by heating at 90°C for 1 min and then at 56°C for 10 min, followed by the addition of 0.4 U/μL Rnasin and 2 mM dithiothreitol. Binding of 30 nM recombinant IMP3 to reanimated RNA was carried out at 30°C for 20 min, and 30 nM let-7g mRNA was subsequently added for 10 min. The acylation reaction by 8 mM 1-methyl-7-nitrosoanhydride (1M7) proceeded for 70 s at room temperature, and the reaction was stopped by the addition of water. Analysis of acylation reactions and dideoxynucleotide sequencing tracks were obtained by reverse transcription initiated by a 5’ end-labeled primer complementary to HMGA2 3’ UTR positions 1,283–1,306 as described previously (Nielsen and Christansen, 1993).

**Whole-Mount RNA In Situ Hybridization**

Whole-mount RNA in situ hybridization was performed as previously described (Hansen et al., 2004). In brief, embryos were fixed overnight in 4% paraformaldehyde at 4°C. After three washes in PBST (PBS plus 1% Tween 20), the embryos were rehydrated in a series of methanol-PBST, bleached in 6% hydrogen peroxide, and treated with proteinase K (Roche). The embryos were pretreated in hybridization buffer containing yeast tRNA and hybridized at 65°C overnight in hybridization buffer containing 1 μg/ml of Hmgag2 or Imp1 digoxigenin-labeled antisense riboprobe. After hybridization, the embryos were washed in RNaseA-containing wash buffer. The transcripts were detected by overnight incubation with digoxigenin antibody (Roche).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.015.

**ACKNOWLEDGMENTS**

We thank Lis Schutt Nielsen, Stine Østergaard, Susanne Smed, and Lena Bjorn Johansson for technical assistance and Dr. Jens Lykke-Andersen for supplying the DCP1a antibody. This study was supported by grants from the Danish Medical and Strategic Research Councils, the Svend Andersen Foundation, the Toyota Foundation, and the Lundbeck Foundation.

Received: July 1, 2013
Revised: January 28, 2014
Accepted: March 6, 2014
Published: April 3, 2014

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