Multiple mechanisms disrupt the let–7 microRNA family in neuroblastoma

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Poor prognosis in neuroblastoma is associated with genetic amplification of MYCN. MYCN is itself a target of let–7, a tumour suppressor family of microRNAs implicated in numerous cancers. LIN28B, an inhibitor of let–7 biogenesis, is overexpressed in neuroblastoma and has been reported to regulate MYCN. Here we show, however, that LIN28B is dispensable in MYCN-amplified neuroblastoma cell lines, despite de-repression of let–7. We further demonstrate that MYCN messenger RNA levels in amplified disease are exceptionally high and sufficient to sponge let–7, which reconciles the dispensability of LIN28B. We found that genetic loss of let–7 is common in neuroblastoma, inversely associated with MYCN amplification, and independently associated with poor outcomes, providing a rationale for chromosomal loss patterns in neuroblastoma. We propose that let–7 disruption by LIN28B, MYCN sponging, or genetic loss is a unifying mechanism of neuroblastoma development with broad implications for cancer pathogenesis.

Carcinogenesis involves multiple genetic and epigenetic events, yet the organizing principles underlying their choreography are poorly understood. MicroRNA (miRNA) deregulation is an important component of this landscape through both oncogenic and tumour-suppressive functions of miRNAs3. Of these, the highly conserved let-7 family has a prominent role in the regulation of embryonic development and maintenance of differentiated tissues and is among the most abundantly expressed miRNAs. It serves as a potent tumour suppressor via post-transcriptional repression of multiple oncogenic messenger RNA (mRNA) targets including RAS, MYC, and HMGA2 (refs 2–4). The let-7 family is downregulated in multiple tumour types and has been causally linked to oncogenesis5,8. Uncovering the mechanisms by which let-7 function is neutralized is therefore critical to both the fundamental understanding of cancer pathogenesis and novel therapies.

Several mechanisms of let–7 disruption have emerged in different contexts. First, its biogenesis can be suppressed by the LIN28B RNA-binding protein11, a highly conserved heterochronic gene implicated in cancer and reported to induce tumours in multiple mouse models including hepatocellular carcinoma, colon cancer, Wilms tumour, and neuroblastoma5–6. Second, competing endogenous RNAs (ceRNAs) have been proposed to sponge miRNAs, including let-7, diluting their activity through competition for miRNAs with sites common to multiple ceRNA species8–11. Third, chromosome loss is a suggested mechanism of let-7 disruption in cancer, as genetic deletion of let-7 is associated with several solid tumours4. The neuroblastoma master oncogene, MYCN, has a 910-nucleotide-long 3′ untranslated region (UTR) containing two let-7 binding sites which are almost perfectly conserved among land vertebrates, suggesting strong functional relevance20–22. Coding sequence mutations in neuroblastoma are rare23,24, whereas chromosome arm gain or loss events are common25,26. The most well-known chromosomal aberration is amplification of the MYCN locus, which occurs in ~25% of all neuroblastomas and largely defines poor prognosis27,28. Other common chromosomal deletions at chromosome arms 3p and 11q are inversely associated with MYCN amplification. The reason for this discordance is unknown.

Here we set out to understand the relationship between MYCN and let–7 in neuroblastoma. A complex relationship emerges between LIN28B activity, a novel ceRNA function of the MYCN 3′ UTR, and let–7 genetic loss, which together present a unifying model of let–7 suppression during neuroblastoma pathogenesis. This model provides an organizing principle for understanding distinct genetic patterning in neuroblastoma, with potential implications for cancer in general.

**LIN28B and let–7 regulate the MYCN 3′ UTR**

LIN28B is highly expressed in human neuroblastoma and its expression correlates with tumour stage, rendering the LIN28B/let–7 axis an attractive target for interrogation (Extended Data Fig. 2a–d). Two recent reports concluded that this pathway plays a critical role in regulating MYCN and neuroblastoma cell growth12,13. To examine the relationship between the MYCN transcript, let–7 and LIN28B, we first transfected non-MYCN amplified neuroblastoma cells with the MYCN open reading frame (ORF), with or without the 3′ UTR carrying intact or mutant let–7 sites (Fig. 1a). The full-length wild-type MYCN transcript produced markedly lower MYCN protein levels than the ORF-only construct. Mutation of the let–7 sites in the 3′ UTR partly rescued MYCN expression, implicating let-7 modulation as an important component of MYCN post-transcriptional regulation (Fig. 1b). Expression of LIN28B suppressed the let-7 family in non-MYCN-amplified neuroblastoma cells and conferred a growth advantage. LIN28B rescued expression of the wild-type 3′ UTR construct, demonstrating that LIN28B can support MYCN expression through let–7 repression in the absence of MYCN amplification (Extended Data Fig. 2e, f and Fig. 1c). However, when we transfected MYCN-amplified cells with a let-7a mimic, we observed decreased MYCN protein levels only above 15- and 80-fold increases in cellular levels of let-7a, respectively.

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suggesting that MYCN was refractory to all but exceedingly high levels of exogenous let-7 (Fig. 1d).

**LIN28B is dispensable in MYCN-amplified cells**

Next, we evaluated the previously reported LIN28B-let-7-MYCN regulatory circuit using published lentiviral short hairpin RNA (shRNA) constructs to knockdown LIN28B in MYCN-amplified neuroblastoma cells and observed comparable suppression of MYCN protein levels and cell growth (Extended Data Fig. 3a, b). We further observed reduced xenograft tumour growth in cells expressing a LIN28B targeting shRNA (Extended Data Fig. 3c). However, we did not observe an appreciable de-repression of let-7 levels upon shRNA-mediated LIN28B knockdown, which is counter to the established model (Extended Data Fig. 3d). Moreover, we were unable to rescue these effects through overexpression of shRNA-resistant LIN28B constructs (Extended Data Fig. 3e, f). Together, these data suggest that the reported effects of the shRNAs on both cell growth and MYCN protein levels might be due to hairpin-induced toxicities.

As an alternative approach to depleting LIN28B, we tested five small interfering RNAs (siRNAs) and found that four both effectively knocked down LIN28B and, as expected, de-repressed let-7 levels (Extended Data Fig. 4a–d). Upon extended serial siRNA transfection, we observed that despite robust LIN28B knockdown and strong de-repression of let-7, MYCN protein levels were unaffected and there was no appreciable effect on cell proliferation (Extended Data Fig. 4e–g).

To exclude the possibility of incomplete knockdown resulting in residual LIN28B activity, we employed Cas9 and four distinct guide RNAs (gRNAs) targeting LIN28B (Extended Data Fig. 4h). We observed robust loss of LIN28B protein with all four gRNA constructs (Fig. 2a, b), indicating efficient disruption of the locus. We did not observe appreciable loss of MYCN protein expression or impaired cell growth, thus corroborating our siRNA-based results (Fig. 2a–d). In addition, the let-7 family was robustly de-repressed, consistent with the existing LIN28B-let-7 model (Fig. 2e, f). These observations indicate that disruption of LIN28B has little net impact on MYCN-amplified neuroblastoma cells.

**Amplified MYCN mRNA is a let-7 sponge**

The persistence of MYCN protein levels despite high levels of transfected let-7a or robust de-repression of let-7 upon LIN28B loss prompted us to explore novel mechanisms of let-7 perturbation. We hypothesized that there might be a ceRNA in MYCN-amplified neuroblastoma cells that serves to sponge let-7. To identify potential ceRNAs, we performed poly-A selected RNA-sequencing (mRNA-seq) on MYCN-amplified (BE(2)C and Kelly) and non-MYCN-amplified (SH-SY5Y) cells. We then determined the relative contribution of let-7 target sites provided by expressed let-7 targets. Interestingly, MYCN itself was by far the most abundant let-7 target mRNA in both BE(2)C and Kelly cells, alone providing 19.3% and 18.5% of the entire cellular let-7 target-site pool, respectively. In contrast, MYCN represented only 0.15% of the let-7 target-site pool in SH-SY5Y cells (Fig. 3a). In fact, MYCN mRNA was the second highest expressed mRNA in both BE(2)C and Kelly cells as opposed to the 5,409th highest in SH-SY5Y, demonstrating an exceptionally high MYCN mRNA level in MYCN-amplified cells (>100-fold increase; Fig. 3b). Other multiple-let-7-site mRNAs such as HMG2A, IMP1, and ARID3B were expressed at much lower levels, together suggesting that MYCN mRNA might itself be the sponge (Extended Data Fig. 5a). This expression pattern was validated by quantitative PCR (qPCR) in a panel of additional cell lines (Extended Data Fig. 5b).

ceRNA relationships were initially defined in part by similar expression levels between RNAs with similar 3′ UTRs. Two recent reports have refined this original precept, suggesting that for a given miRNA family, the miRNA-miRNA-target ratio is a major determinant of how effectively a ceRNA can impact the function of a miRNA family. At low ratios, miRNAs are sensitive to moderate levels of ceRNAs, whereas highly expressed miRNAs with high ratios are difficult to sponge, requiring very high levels of ceRNA. Therefore, we assessed total copies per cell of both MYCN mRNA and the let-7 family in BE(2)C and Kelly cells through quantified mRNA-seq and small-RNA sequencing (sRNA-seq) (Extended Data Fig. 6).

We calculated 13,255 and 10,615 MYCN mRNA copies per BE(2)C and Kelly cell, respectively, resulting in 26,511 and 21,231 copies per cell of both MYCN mRNA copies per BE(2)C and Kelly cell, respectively, resulting in 26,511 and 21,231.
amplified mRNA. Interestingly, let-7a was the most highly expressed let-7 family member in both cell types, accounting for over half of all let-7 molecules. These observations were confirmed by spike-in qPCR-based quantification of both MYCN and let-7 (Extended Data Fig. 7a, b). Even upon LIN28B knockout, the MYCN:let-7 site:let-7 ratio is 1.35 in BE(2)C and 1.78 in Kelly, which remain favourable for ceRNA activity (Extended Data Fig. 7c).

To test the capacity of MYCN mRNA to serve as a let-7 sponge, we co-transfected BE(2)C cells with a series of luciferase constructs containing the 3′ UTRs of several representative let-7 targets and control or MYCN ORF targeting siRNA (Extended Data Fig. 8a, b). Luciferase ratios of all constructs except for empty vector controls and the let-7-site-mutated MYCN-3′ UTR were significantly reduced by either MYCN knockdown or let-7a transfection (Fig. 3d), suggesting that the endogenous MYCN-3′ UTR sponges steady-state levels of let-7. We then tested the sufficiency of the MYCN-3′ UTR to de-repress let-7 targets through sponging of a let-7a mimic. We co-transfected the above luciferase reporter constructs with chimaeric RFP:MYCN-3′ UTR constructs and assayed luciferase activity (Extended Data Fig. 8c, d). The let-7 target constructs were rescued when co-transfected with wild-type but not let-7-site-mutant MYCN-3′ UTR (Extended Data Fig. 8e).

In addition, exogenous MYCN-3′ UTR was sufficient to enhance MYCN protein expression itself in SK-N-AS cells (Extended Data Fig. 8f).

Next we tested whether endogenous let-7 targets are similarly affected. Upon MYCN knockdown, protein levels of DICER1, HK2, IMP1, and LIN28B were reduced, while neither mRNA nor let-7 levels were significantly changed (Extended Data Fig. 9a–c). Concurrent let-7 inhibition rescued expression of the four targets, supporting post-transcriptional suppression through let-7 upon MYCN knockdown that is consistent with MYCN mRNA serving as a let-7 sponge (Extended Data Fig. 9a). Further, these targets were not reduced at the protein level upon LIN28B knockout, which is consistent with this model (Extended Data Fig. 9d).

Lastly, we analysed global let-7 target expression in response to depletion of the endogenous MYCN 3′ UTR. To specifically assess the role of the 3′ UTR, we transfected BE(2)C cells expressing a MYCN ORF transgene with a MYCN 3′ UTR-targeting siRNA (Fig. 3e). Although we did not see a global reduction of let-7 targets as a whole (Extended Data Fig. 9e), we did observe significantly lower expression of let-7 targets with three or more total sites or more than one octamer let-7 site in their 3′ UTRs, which together define the most sensitive let-7 targets (Fig. 3f). Given the regulation of the MYCN 3′ UTR by multiple miRNAs and unknown kinetics of how let-7 activity is restored after the removal of a ceRNA, we further challenged siCon and siMYCN cells with a modest amount of let-7a mimic, increasing cellular let-7 levels approximately eightfold (Extended Data Fig. 9f). We then observed significantly reduced expression across all categories of let-7 targets in siMYCN cells, consistent with increased sensitivity to let-7 in the absence of the MYCN 3′ UTR (Fig. 3g and Extended Data Fig. 9g).

**Chromosomal loss of let-7 in neuroblastoma**

While neuroblastoma has a low mutation rate, chromosome arm gain and loss is frequent23,24. Two of the most common chromosomal losses in neuroblastoma, chromosome arm 3p (Chr3p, ~33% incidence) and Chr11q (~45% incidence), often occur together and seldom with MYCN amplification23,25,26. (Fig. 4a and Extended Data Fig. 10). Upon analysis of Chr3p and Chr11q, we noted that the Chr3p-loss smallest region of overlap spans from 3p25.3 to 3p14.3 (ref. 37), placing let-7g...
within the smallest region of overlap and resulting in its loss whenever Chr3p is lost. In addition, the most common breakpoint of Chr11q immediately proximal to the let-7a2 locus, resulting in loss of let-7a2 in virtually all Chr11q deletions\(^3\) (Fig. 4b). Moreover, loss of Chr11q in neuroblastoma results in lower overall let-7a levels despite loss of only one of three distinct let-7a loci\(^3\). These observations suggest that let-7 genetic disruption may be selected for in neuroblastoma.

A model in which amplification MYCN sequesters mature let-7 would predict that selective pressure to genetically lose let-7 would be relieved in MYCN-amplified disease. Chr3p (let-7g) and Chr11q (let-7a) loss patterns are indeed consistent with such a model. To investigate whether the extended let-7 family (Fig. 4b) follows this loss pattern, we expanded our analysis to all eight let-7 genetic loci in 202 neuroblastomas by array comparative genomic hybridization (aCGH). We created a heat-map of copy number estimates for each miRNA locus to compare MYCN-amplified with non-amplified copy number values and observed a significant difference for six let-7 loci (Fig. 5a, b top). At least one let-7 family member was lost in 63.4% of non-amplified tumours and in only 16.7% of amplified (Fig. 5b top), resulting in average let-7 copy number changes of \(-1.94\) and \(-0.36\) per tumour, respectively (Fig. 5b bottom). This pattern of copy number loss for let-7 is distinct from the unrelated miR-103a family (Fig. 5b), suggesting that the let-7 pattern is not reflective of general chromosomal patterning.

The most commonly lost were let-7a2, let-7f2, and let-7g, whereas let-7a3/7b and let-7i were not significantly lost in any tumour subset. We reasoned that loss frequency might relate to initial expression level. To interrogate this possibility, we used publically available sRNA-seq data to examine the relative expression levels of mature let-7 in 12 distinct primary and tumour cell lines that have intact let-7 loci (Extended Data Fig. 10b). We observed that let-7a, let-7f, and let-7g are present at higher relative levels than other let-7 family members, which mirrors the copy number loss patterns in non-MYCN-amplified neuroblastoma (Fig. 5c). MiR-100, the miRNA-cluster partner of let-7a2, is more highly expressed than most let-7s and their cluster partners, suggesting that...
the bulk of the let-7a reads may come from the let-7a2/miR-100 locus (Fig. 5c).

A limitation of sRNA-seq is that it cannot distinguish between loci that produce the same mature miRNA. Family members of let-7 are coordinately transcribed as part of larger host transcripts from which they are then processed, which allows for locus-specific expression analysis3,39,40 (Extended Data Fig. 10c). We therefore analysed relative expression levels of let-7 host transcripts in six primary and tumour cell lines. Host transcript levels for let-7a2, let-7j2, and let-7g were significantly higher than for other let-7 host transcripts (Extended Data Fig. 10d and Fig. 5d), reflecting the pattern of most frequent locus loss in non-amplified disease. In addition, analysis of existing human neuroblastoma mRNA-seq and microarray data sets revealed lower expression of the let-7a2, let-7j2, and let-7g host transcripts in non-amplified compared with MYCN-amplified tumours, which is consistent with observed patterns of copy number loss in our aCGH data set (Fig. 5e and Extended Data Fig. 10e). Further, MYCN and let-7 expression are negatively correlated in non-amplified disease, underscoring the importance of let-7 disruption in the absence of the MYCN ceRNA34. These data may collectively explain both preferential loss of certain let-7 loci and common patterns of chromosomal loss in neuroblastoma.

Further emphasizing the significance of let-7 suppression is the observation that non-MYCN-amplified neuroblastoma patients had significantly worse overall survival if there was a let-7 copy number loss event (Fig. 5f top). In the rare case where MYCN amplification and let-7 copy number loss occurred together, overall survival was dramatically reduced relative to the already-poor prognosis of MYCN amplification (Fig. 5f bottom), suggesting a deleterious synergy between two powerful but typically exclusive mechanisms of functional let-7 disruption.

Of note, one of the tumours in the aCGH data set had genetic amplification of LIN28B (copy number = 23; Fig. 5a). This tumour patterned closely with MYCN-amplified tumours with regard to net let-7 loss and tumour stage (IV) despite being 2n for MYCN. This may represent a case where LIN28B significantly contributed to neuroblastoma through let-7 suppression, similar to the reported mouse model of murine-Lin28b-driven neuroblastoma42.

Discussion

The known functionality of LIN28B, together with the patterns of genetic deletion of let-7 and amplified MYCN ceRNA (aceRNA) activity described here, establish that neuroblastoma employs multiple mechanisms to neutralize let-7, placing let-7 disruption at the centre of neuroblastoma pathogenesis. We thus propose that let-7 biogenesis and function are targeted in neuroblastoma by several disparate mechanisms: high frequency genetic loss, LIN28B activity, or MYCN aceRNA (Extended Data Fig. 10f). This model has implications for our understanding of neuroblastoma pathogenesis, disease modelling, and the rational design of therapeutic strategies, and may represent a more general feature of human cancer.

First, our model offers a plausible explanation for the uniquely high MYCN mRNA levels in amplified neuroblastoma, which enable both robust expression of MYCN protein and adequate copies of a ceRNA sufficient to impair the function of a highly expressed miRNA such as let-7. Questions may remain about whether an observed >100-fold increase in MYCN mRNA, which increases the total let-7 target sites across the cellular pool of mRNAs by only approximately 25%, is sufficient to mediate a ceRNA effect on let-7. However, our functional data based on loss of the MYCN 3′ UTR, including candidate reporter analysis of let-7-site-containing 3′ UTRs and global let-7-target mRNA-seq analysis (Fig. 3d–g), suggest that MYCN mRNA may be a preferred target that in abundance can sequester and impair let-7. Further, in tumours lacking MYCN amplification, our model suggests that selective pressure to disrupt let-7 explains the well-known, yet unresolved, patterns of MYCN-amplification-independent chromosome 3p and 11q loss. AceRNA function of MYCN mRNA also accounts for the dispensability of LIN28B in MYCN-amplified cell lines, suggesting that LIN28B may serve a redundant let-7 suppressive role.

Our findings suggest that highly expressed 3′ UTRs contribute to miRNA deregulation in cancer, and therefore both coding and non-coding functions of oncogenic mRNAs should be considered in animal tumour modelling. For example, both the TH-MYCN and LSL-MYCN;Dbh-iCre models of murine neuroblastoma overexpress the MYCN ORF and lack 3′ UTRs. Notably, the TH-MYCN model has similar patterns of let-7a2, let-7j2, and let-7g genetic loss as non-amplified human disease and both models broadly downregulate the let-7 family34,41, further suggesting that let-7 disruption is important even in the presence of MYCN protein expression. Moreover, a recent report demonstrates that high-level expression of the BRAF pseudogene, which contains a functional 3′ UTR but does not encode a protein, is sufficient to induce lymphoma in mice42. Consequently, full-length mRNA transgenes may yield more accurate genetic modelling of human tumours in animals.

Lastly, our model establishes let-7 restoration as a key therapeutic goal in neuroblastoma. There are few neuroblastoma-specific therapies, and attempts to directly target MYCN have met with little success, despite efforts spanning the past 20 years (refs 25, 26, 43). The fact that MYCN mRNA has such a strong functional connection to let-7 exposes a valuable opportunity to target MYCN itself and provides hope of delivering disease-specific therapy to the worst prognostic class of neuroblastoma.

We show here that disparate modes of let-7 suppression are selectively and inversely related in neuroblastoma. Given that both oncogenic amplification and disruption of let-7 biogenesis appear to play central roles in multiple cancer types15,44–47, our model may provide a novel organizing principle by which to consider and interrogate genetic events in a broad range of tumours.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Croce, C. M. Causes and consequences of microRNA dysregulation in cancer. Nature Rev. Genet. 10, 704–714 (2009).
2. Johnson, S. M. et al. RAS is regulated by the let-7 microRNA family. Cell 120, 635–647 (2005).
3. Sampson, V. B. et al. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res. 67, 9762–9770 (2007).
4. Mayr, C., Hemann, M. T. & Bartel, D. P. Disrupting the pairing between let-7 and Hmg2a enhances oncogenic transformation. Science 315, 1576–1579 (2007).
5. Lu, J. et al. MicroRNA expression profiles classify human cancers. Nature 435, 834–838 (2005).
6. Boyerinas, B., Park, S.-M., Hau, A., Murmann, A. E. & Peter, M. E. The role of let-7 in cell differentiation and cancer. Endocr. Relat. Cancer 17, F19–F36 (2010).
7. Gurtan, A. M. & Sharp, P. A. The role of miRNAs in regulating gene expression networks. J. Mol. Biol. 425, 3582–3600 (2013).
8. Blandino, G. et al. Tumor suppressor microRNAs: a novel non-coding alliance against cancer. FEBS Lett. 588, 2639–2652 (2014).
9. Viswanathan, S. R., Daley, G. Q. & Gregory, R. I. Selective blockade of microRNA processing by Lin28. Science 320, 97–100 (2008).
10. Nguyen, L. H. et al. Lin28b is sufficient to drive liver cancer and necessary for its maintenance in murine models. Cancer Cell 26, 248–261 (2014).
11. Viswanathan, S. R. et al. Lin28 promotes transformation and is associated with advanced human malignancies. Nature Genet. 41, 843–848 (2009).
12. Molenar, J. J. et al. LIN28 induces neuroblastoma and enhances MYCN levels via let-7 suppression. Nature Genet. 44, 1199–1206 (2012).
13. Diskin, S. J. et al. Common variation at 6q16 within HACE1 and LIN28B influences susceptibility to neuroblastoma. Nature Genet. 44, 1126–1130 (2012).
14. Madison, B. B. et al. LIN28B promotes growth and tumorigenesis of the intestinal epithelium via Let-7. Genes Dev. 27, 2233–2245 (2013).
15. Urbach, A. et al. Lin28 sustains early renal progenitors and induces Wilms tumorigenesis. Genes Dev. 28, 971–982 (2014).
16. Tu, H. C. et al. LIN28 cooperates with Wnt signaling to drive invasive intestinal and colorectal adenocarcinoma in mice and humans. Genes Dev. 29, 1074–1086 (2015).
17. Tay, Y., Rinn, J. & Pandolfi, P. P. The multilayered complexity of ceRNA cross-talk and competition. Nature 505, 344–352 (2014).
18. Poliseno, L. et al. A coding-independent function of gene and pseudogene miRNAs regulates tumour biology. Nature 465, 1033–1038 (2010).
19. Cesana, M. & Daley, G. Q. Deciphering the rules of ceRNA networks. Proc. Natl Acad. Sci. USA 110, 7112–7113 (2013).
20. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20 (2005).
21. Melton, C., Judson, R. L. & Blielloch, R. Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. Nature 463, 621–626 (2010).
22. Baeyens, K. J., De Bondt, H. L., Pardi, A. & Holbrook, S. R. A curved RNA helix incorporating an internal loop with G·A and A·G non-Watson–Crick base pairing. Proc. Natl Acad. Sci. USA 93, 12851–12855 (1996).
23. Pugh, T. J. et al. The genetic landscape of high-risk neuroblastoma. Nature Genet. 45, 279–284 (2013).
24. Molenaar, J. J. et al. Sequencing of neuroblastoma identifies chromothripsis and defects in neurotogenesis genes. Nature 483, 589–593 (2012).
25. Barone, G., Anderson, J., Pearson, A. D. J., Petrie, K. & Chesler, L. New strategies in neuroblastoma: therapeutic targeting of MYCN and ALK. Clin. Cancer Res. 19, 5814–5421 (2013).
26. Maris, J. M. Recent advances in neuroblastoma. N. Engl. J. Med. 362, 2202–2211 (2010).
27. Seeger, R. C. et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N. Engl. J. Med. 313, 1111–1116 (1985).
28. Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E. & Bishop, J. M. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224, 1121–1124 (1984).
29. Ala, U. et al. Integrated transcriptional and competitive endogenous RNA networks are cross-regulated in permissive molecular environments. Proc. Natl Acad. Sci. USA 110, 7164–7169 (2013).
30. Denzler, R., Agarwal, V., Stefano, J., Bartel, D. P. & Stoffel, M. Assessing the ceRNA hypothesis with quantitative measurements of miRNA and target abundance. Mol. Cell 54, 766–776 (2014).
31. Boisson, A. D., Zamudio, J. R. & Sharp, P. A. Endogenous miRNA and target concentrations determine susceptibility to potential ceRNA competition. Mol. Cell 56, 347–359 (2014).
32. Agarwal, V., Bell, G. W., Nam, J.-W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian miRNAs. elife 4, http://dx.doi.org/10.7554/eLife.05005 (2015).
33. Bartel, D. P. MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233 (2009).
34. Beckers, A. et al. MYCN-targeting miRNAs are predominantly downregulated during MYCN-driven neuroblastoma tumor formation. Oncotarget 6, 5204–5216 (2015).
35. Maris, J. M. et al. Allelic deletion at chromosome bands 11q14-23 is common in neuroblastoma. Med. Pediatr. Oncol. 36, 24–27 (2001).
36. Breen, C. J., O’Meara, A., McDermott, M., Mullarkey, M. & Stallings, R. L. Coordinate deletion of chromosome 3p and 11q in neuroblastoma detected by comparative genomic hybridization. Cancer Genet. Cyto-genet. 120, 44–49 (2000).
37. Ejeskär, K., Abrutani, H., Abrahamsson, J., Kogner, P. & Martinsson, T. Loss of heterozygosity of 3p markers in neuroblastoma tumours implicate a tumour-suppressor locus distal to the FHT gene. Br. J. Cancer 77, 1787–1791 (1998).
38. Bray, I. et al. Widespread dysregulation of miRNAs by MYCN amplification and chromosomal imbalances in neuroblastoma: association of miRNA expression with survival. PloS ONE 4, e7850 (2009).
39. Chiang, H. R. et al. Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. Genes Dev. 24, 992–1009 (2010).
40. Roush, S. & Slack, F. J. The let-7 family of microRNAs. Trends Cell Biol. 18, 505–516 (2008).
41. Rakheja, D. et al. Genome-wide array CGH analysis of murine neuroblastoma reveals distinct genomic aberrations which parallel those in human tumors. Cancer Res. 63, 5266–5273 (2003).
42. Karreth, F. A. et al. The BRAF pseudogene functions as a competitive endogenous RNA and induces lymphoma in vivo. Cell 161, 319–332 (2015).
43. Maris, J. M., Hogarty, M. D., Bagatell, R. & Cohn, S. L. Neuroblastoma. Lancet 369, 2106–2120 (2007).
44. Pugh, T. J. et al. Exome sequencing of pleuropulmonary blastoma reveals frequent biallelic loss of TP53 and two hits in DICER1 resulting in retention of 5p-derived miRNA hairpin loop sequences. Oncogene 33, 5299–5302 (2014).
45. Ejeskär, K. et al. Somatic mutations in DROSHA and Dicer1 impair microRNA biogenesis through distinct mechanisms in Wilms tumours. Nature Commun. 5, 4802 (2014).
46. Rui, W. et al. Genome-wide identification of genes with amplification and/or fusion in small cell lung cancer. Genes Chromosom. Cancer 52, 802–816 (2013).
47. Thériault, B. L., Dimaras, H., Gallie, B. L. & Corson, T. W. The genomic landscape of retinoblastoma: a review. Clin. Experiment. Ophthalmol. 42, 33–52 (2014).

Supplementary Information is available in the online version of the paper.

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**METHODS**

**Cell culture.** BE(2)C (ATCC CRL-2268), PA-1 (ATCC CRL-1572), IMR90 (ATCC CRL-186), SK-N-AS (ATCC CRL-2137), SH-SY5Y (ATCC CRL-2266), 293T (ATCC 11268), SK-N-DZ (ATCC CRL-2149), and Kelly cells (Sigma S2110441-1VL) were maintained in 1:1 DMEM/F12 MEM media with 10% inactivated fetal calf serum, 1 mg ml⁻¹ penicillin, and 1 μl⁻¹ streptomycin. All cell lines were purchased for the purposes of this study, and are not among commonly misidentified cell lines (according to the International Cell Line Authentication Committee and tested negative for mycoplasma contamination)

**Plasmids.** Turbo-RFP, LIN28B, and MYCN (ORF) cDNAs were subcloned into the pCDNA3.1 expression vector (Invitrogen). The MYCN 3' UTR was cloned from BE(2)C cDNA and subcloned into pCDNA3.1-MYCN to create pCDNA3.1-MYCN3' UTR. RshRNA-resistant-LIN28B and pCDNA3.1-MYCN3' UTR-let-7-site-mutant vectors were made using a QuikChange site-directed-mutagenesis kit (Stratagene) on pCDNA3.1-LIN28B and pCDNA3.1-MYCN3' UTR constructs, respectively. Wild-type and mutant MYCN 3' UTRs were subcloned into pCDNA3.1-RFP and psiCHECK2 to create pCDNA3.1-RFP-MYC3' UTRwt and pCDNA3.1-RFP-MYC3' UTRmut as well as psiCHECK2-MYCNwt and psi-CHECK2-MYCNmut. psisin1::Dicer1, psisin1::GFP2B1, and psisin1::Hmga2 were gifts from D. Bartel48 (Addgene plasmids 21649, 21639, and 14785). psiCHECK2-8xlet-7 was a gift from Y. Tomari49 (Addgene plasmid 20931).

**siRNA/let-7 mimic transfections.** BE(2)C and Kelly cells were reverse transfection using Lipofectamine 2000 (Life Technologies) into six-well plates using the appropriate siRNA or miRNA mimics (described below). Cells were harvested at time-points described for analysis by western blotting or qPCR. Growth assays were performed similarly, but in 96-well plates followed by time-point-specific BrdU growth assay. Global let-7 target analysis was as follows: BE(2)C:MYCN-ORF cells co-transfected with control or MYCN 3'UTR-2 siRNA and either control or let-7a mimRNA mimics. LNCaP cells were transfected 48 h after transfection. Control siRNA (Life Technologies 439846), LIN28B siRNAs were as follows: ORF1 (Life Technologies 4392420, identifier s5247), ORF2 (Life Technologies 4392420, identifier s5247), 5' UTR (GE Dharmacon Custom LIN28B_NM_001043471 Duplex siRNA, ON-TARGET Plus, sense: 5'-ACU GGA GAG AGG AGA AAA AUU-3', antisense: 5'-UUU CUC UCC UCU CUC CAG UUU-3', 3' UTR (GE Dharmacon J-02884-0012-00), 3' UTR (GE Dharmacon Custom LIN28B_NM_001043171 Duplex siRNA, ON-TARGET Plus, sense: 5'-CAA CUG UGA UUG GAA UAA AUU-3', antisense: 5'-AUC CUC ACA AUC AGU GUU GUU-3'). MYCN siRNAs: ORF-1 (Life Technologies 4392420, identifier s1935), ORF-2 (Life Technologies 4392420, identifier s1934). Control miRNA mimic (Life Technologies 4463209), let-7a miRNA mimic (Life Technologies 4464066, identifier MC10050), let-7a inhibitor (Life Technologies 4463208, identifier MH10050).

**Luciferase assays.** For the MYCN 3' UTR loss of function assays, BE(2)C cells were reverse co-transfected using Lipofectamine 2000 in quadraplicate into 96-well plates with the appropriate luciferase vector and either control siRNA, MYCN siRNA, or let-7a mimic. Sixty hours after transfection, luciferase activity was assayed using the Dual Luciferase Reporter Assay System (Promega). For the MYCN 3' UTR gain of function assays, 293T cells were seeded into 96-well plates with the appropriate luciferase vector, MYCN 3' UTR overexpression vector, and either control miRNA mimic or let-7a mimic. Luciferase activity was measured 24 h after transfection as described above.

**Immunohistochemistry.** Immunohistochemistry was performed on human tumour tissue sections as previously described using anti-LIN28B antibody (Cell Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2; Immunohistochemical and Molecular Signaling 4196) at a 1:400 dilution. Patient samples were obtained through Boston Children’s Hospital IRB-CRS08-09-0429-2. 

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5′-end-scrambled let-7a and let-7i spikes, we sought to minimize the effects of secondary structure bias known to exist during sRNA-seq library preparations, which can have significant effects on the relative efficiency of reads produced between different microRNAs11 (Extended Data Fig. 6). A total of six synthetic spikes were used. Disparate reads per million observed among the equimolar small RNA spikes (that is, the miR-Neg, LET7A2, LET7I spikes have much higher read counts than equimolar MIR17, miRNegDH1, and miRNegDH2 spikes) demonstrate both the need to use spikes similar to the miRNA-of-interest to accurately determine copies per cell and the risk inherent in using a single spike to determine copies per cell for all miRNAs. The use of multiple miRNA-specific spikes improves upon previous miRNA quantification strategies using a single control miRNA to quantify all miRNAs8 by limiting potential disparate read efficiencies between a single spike and the miRNA of interest. Difficulty in calculating relative expression of disparate miRNAs within a single data set because of such variable read efficiencies can therefore be mitigated through the use of spike-ins that closely resemble each miRNA of interest.

CRISPR/Cas9. Cas9/gRNA co-expressing lentiviral constructs (lentiCRISPRv2) were generated and lentiviral particles were produced as previously described13,14, using protocols and gRNA design tools from http://www.genome-engineering.org. Puromycin selection began 24 h after lentiviral infection of BE(2)C and Kelly cells. Experiments were typically completed within 3 weeks of initial infection. Oligonucleotides used for gRNA cloning were as follows: LIN28B exon 2: CAC CGC ATC TGG AAT ATC CAA G, AAA CCT TGG ATA TTC TGC TGG A, AAA CTC CAT GAA TAT TCT GCT C; LIN28B exon 3.1: CAC CGC AGA GCA AAC TAT TCA TGG A, AAA CCT TGG ATA TTC CAG TCG LIN28B exon 3.2: CAC CGA ATG ATT ACC TAT CCT C; AAA CAA GGG AGA TAG GTA ATC ATT C; LIN28B exon 4: CAC CGC CTT GAT GCT ACA ACT G; AAA ACA GGT GTA GCA TCT TAC AGG C. Cas9/gRNA constructs: lentiCRISPRv2, lentiCRISPR:EGFPs-gRNA-1, and lentiCRISPR:EGFPs-gRNA-2 were gifts from F. Zhang53,54 (Addgene plasmids 52961, 51760, and 51761).

shRNA. Lentiviral particles were prepared as previously described11; pLKO.1 short hairpin expression constructs (Sigma Mission shRNA): LIN28B shRNAs (sh1: TRCN0000144508, sequence 5′-TCT ACA AGG C; sh2: TRCN0000122599, sequence 5′-TCT ACA AGG C). Control vector (pShc001; no insert).

Xenografts. BE(2)C and SK-N-DZ cells were infected with either SHC001 or TRCN0000122599 plKO.1 lentivirus, then puromycin selected for 48 h. One and a half million infected cells were injected subcutaneously into female Rag2 knock-out (c57bl/6, 8-week-old) immune-deficient mice. Three weeks after injection, mice were killed and tumours were removed and weighed. This procedure was approved by the Boston Children's Hospital Institutional Animal Care and Use Committee under protocol 15-12-3071R, which limited xenograft tumour size to less than 2.0 cm.

aCGH analysis. Preparation of the 202 neuroblastoma aCGH data set has been previously described15,16. MicroRNA-containing loci were analysed for gain or loss as previously described17. Statistical significance between MTCN-amplified and non-amplified tumours was determined using a t-test with Welch’s adjustment on original copy number values. Kaplan–Meyer curve generation and analysis was done using GraphPad Prism software.

ENCODE RNA-Seq data sets. Mature let-7 expression data for 12 cell types were obtained from whole-cell small RNA-Seq ENCODE/CHSL data sets, and let-7 host transcript expression data from six cell types were obtained from RNA-seq ENCODE data sets on the University of California, Santa Cruz Genome Browser18 (http://genome.ucsc.edu/). Expression levels were determined relative to let-7c and let-7h host transcript levels, respectively.

R2 database. Human neuroblastoma patient microarray and RNA-seq data sets were obtained from the R2: microarray analysis and visualization platform (http://r2.amc.nl) and analysed using GraphPad Prism software. Significance was determined by t-test with Welch’s adjustment. Data sets used were Kozak (GEO accession number GSE45547) and SEQC (GEO accession number GSE62564).

Statistical analysis. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. Unless otherwise noted, all experiments were performed at least three times independently. Statistical tests used are identified in each figure legend. P values less than 0.05 were considered significant.

48. Mayr, C. & Bartel, D. P. Widespread shortening of 3′ UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell 138, 673–684 (2009).

Iwasaki, S., Kawamata, T. & Tomari, Y. Drosophila argonaute1 and argonaute2 employ distinct mechanisms for translational repression. Mol. Cell 34, 58–67 (2009).

Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. Nature Methods 12, 357–360 (2015).

Hatner, M. et al. RNA-ligase-dependent biases in miRNA representation in deep-sequenced small RNA cDNA libraries. RNA 17, 1697–1712 (2011).

Patro, R., Duggal, G. & Kingsford, C. Salmon: accurate, versatile and ultrafast quantification from RNA-seq data using lightweight-alignment. bioRxiv http://dx.doi.org/10.1101/021592 (2015).

Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. Nature Methods 11, 783–784 (2014).

Shalem, O. et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343, 84–87 (2014).

Spitz, R. et al. Oligonucleotide array-based comparative genomic hybridization (OaCGH) of 90 neuroblastomas reveals aberration patterns closely associated with relapse pattern and outcome. Genes Chromosom. Cancer 45, 1130–1142 (2006).

Thiessen, J. et al. Chromosome 17q17q gain and unaltered profiles in high resolution array-CGH are prognostically informative in neuroblastoma. Genes Chromosom. Cancer 53, 639–649 (2014).

Rushlow, D. E. et al. Characterisation of retinoblastomas without RB1 mutations: genomic, gene expression, and clinical studies. Lancet Oncol. 14, 327–334 (2013).

Kont, W. J. et al. The human genome browser at UCSC. Genome Res. 12, 996–1006 (2002).
Extended Data Figure 1 | MYCN is a highly conserved let-7 target.

**a**, Schematic of human MYCN ORF and 3′ UTR, indicating let-7 sites 1 and 2 and their approximate location. **b**, Predicted base pairing patterns of let-7a, let-7f, and let-7g with MYCN let-7 sites 1 and 2. A–G base pairs, common in RNA, are represented by an asterisk. **c**, Alignments of let-7 sites 1 and 2 in 100 vertebrate MYCN 3′ UTRs (ENCODE, https://genome.ucsc.edu/ENCODE/).
Extended Data Figure 2 | LIN28B expression and function in neuroblastoma. a, MYCN, LIN28B, and LIN28A mRNA expression levels in neuroblastoma (n = 649; see Source Data (ED Fig 2) in Supplementary Information). b, Immunoblot for indicated proteins in human embryonic carcinoma cells (PA1), normal human fibroblasts (HF), SK-N-SH (SH), SK-N-AS (AS), SK-N-F1 (F1), BE2C (BE), SK-N-DZ (DZ), Kelly (Ke), and human chronic myeloid leukaemia cells (K5). For gel source data, see Supplementary Figures. c, Representative LIN28B immunohistochemical staining of human neuroblastoma by stage (left), percentage LIN28B positive neuroblastoma by disease stage (right); (n = 36). GNB, ganglioneuroblastoma. d, LIN28B expression by neuroblastoma stage (n = 64; Source Data (ED Fig 2)). e, Immunoblot for LIN28B in inducible LIN28B SH-SY5Y cells and GFP- or LIN28B-expressing SK-N-AS cells (left) and corresponding qPCR analysis of relative let-7 family levels (right) (mean plus s.e.m. of three independent experiments shown). f, Relative growth rate (BrdU incorporation, right) of SH-SY5Y and SK-N-AS neuroblastoma cells from d (*P < 0.05, n = 3 independent experiments).
Extended Data Figure 3 | Short hairpin knockdown of LIN28B in neuroblastoma. a, Immunoblot for indicated proteins MYCN and LIN28B in MYCN-amplified cells infected with LIN28B targeting lentiviral shRNAs. For gel source data, see Supplementary Figures. b, Cell proliferation analysis of cells described in a (n = 3 independent experiments). c, Average tumour size of human–mouse subcutaneous xenograft tumour analysis 3 weeks after injection of 2 × 10^6 cells infected with a LIN28B targeting lentiviral shRNA (n = 6 mice for BE(2)C, n = 3 mice for SK-N-DZ; Supplementary Figures and Source Data (ED Fig 3)). d, qPCR analysis of let-7a, let-7b, and let-7i levels in cells described in a (mean plus s.e.m. of three independent experiments shown). e, Cell proliferation analysis of BE(2)C cells stably expressing red fluorescence protein (RFP), Flag-tagged LIN28B ORF, or shRNA resistant Flag-tagged LIN28B (LIN28B shRes) infected with LIN28B lentiviral shRNAs targeting the LIN28B 3′ UTR (ShL28B-UTR) or the LIN28B open-reading frame (ShL28B-ORF). Cell counts were performed 7 days after lentiviral shRNA infection (mean plus s.e.m. of three independent experiments shown). f, Immunoblot for indicated proteins in cells described in e. For gel source data, see Supplementary Figures.
Extended Data Figure 4 | Small interfering RNA knockdown of LIN28B in neuroblastoma. a, Schematic of approximate siRNA target sites within the LIN28B mRNA. b, qPCR analysis of LIN28B mRNA levels in BE(2)C cells 48 h after transfection with the indicated LIN28B targeting siRNAs (mean of two independent experiments shown). c, Immunoblot analysis of LIN28B in cells from a. For gel source data, see Supplementary Figures. d, qPCR analysis of indicated let-7 levels in cells from a (mean of two independent experiments shown). e, Immunoblot analysis of MYCN and LIN28B in serially transfected MYCN-amplified cells for 6 or 9 days. Identical transfections were performed on days 0, 3, and 6. For gel source data, see Supplementary Figures. f, Day 9 qPCR analysis of the let-7 family in the cells from a (n = 3 independent experiments, mean plus s.e.m. shown). g, Cell growth analysis of day 0 to day 6 cells from a (BrdU incorporation, n = 3 independent experiments, mean plus s.e.m. shown). h, Lentiviral CRISPR-Cas9/LIN28B gRNA strategy targeting LIN28B at four distinct exon/intron junctions used in b–g.
Extended Data Figure 5 | Relative levels of let-7 targets in neuroblastoma. a, mRNA-seq let-7 target table (as percentage let-7 target-site pool). b, qPCR analysis of indicated let-7 targets in neuroblastoma cells, PA1 embryonic carcinoma cells (EC), and normal human fibroblasts (hFib). Expression relative to β-ACTIN (ΔCT method) (mean of two biological replicates shown).
Extended Data Figure 6 | Heat map of let-7 and small RNA spike reads. Heat map of three BE(2)C and three Kelly sRNA-seq samples depicting the relative reads per million of the let-7 family, miR-17, and the six small RNA spikes added in equimolar amounts per sample (spikes miR-Neg, LET7A2, and LET7I were used to determine let-7 copies per cell from the small RNA sequencing data set). RPM, reads per million.
Extended Data Figure 7 | qPCR quantification of MYCN and let-7 copies per cell. 

a, Total let-7 sites per cell provided by MYCN mRNA in BE(2)C, Kelly, normal human fibroblasts (NHF), and embryonic carcinoma cells (EC) (mean plus s.e.m. of three biological replicates shown). 
b, Total let-7 copies per cell in cells from a, presented as stacked graphs of all let-7 family members (mean of three biological replicates shown). 
c, Total let-7 copies per cell in wild-type or LIN28B knockout BE(2)C and Kelly cells, presented as stacked graphs of all let-7 family members (values derived from let-7 copies per cell in b and average let-7 fold change described in Fig. 2f, g).
Extended Data Figure 8 | Luciferase reporter and gain of function constructs. a, Luciferase constructs used in the luciferase assays in Fig. 3d and Extended Data Fig. 8e. b, Schematic of the luciferase transfection protocols used in Fig. 3d. c, Schematic of the luciferase protocol used in Extended Data Fig. 8e. d, pcDNA3.1 constructs used in Extended Data Fig. 8e, f. e, Top: relative luciferase ratio in 293T cells co-transfected with the indicated 3′ UTR luciferase and pcDNA3.1 vectors in the presence of either a control miRNA or let-7a mimic. Mean of four independent experiments plus s.e.m. shown (*P < 0.05 relative to empty vector, unpaired t-test). f, Immunoblot analysis of MYCN in SK-N-AS cells stably expressing a MYCN ORF + 3′ UTR transgene and transfected with the indicated pcDNA3.1 vector. For gel source data, see Supplementary Figures.

Bottom: relative luciferase ratio in 293T cells co-transfected with the indicated 3′ UTR luciferase and pcDNA3.1 vectors in the presence of either a control miRNA or let-7a mimic. Mean of four independent experiments plus s.e.m. shown (*P < 0.05 relative to empty vector, unpaired t-test).
Extended Data Figure 9 | MYCN mRNA sponges let-7. a, Immunoblot analysis of indicated proteins in BE(2)C cells transfected for 2.5 days with control, MYCN-1 (M1), or MYCN-2 (M2) siRNA and either control microRNA or let-7a inhibitor. For gel source data, see Supplementary Figures. b, qPCR analysis of DICER1, HK2, IMP1, LIN28B, and MYCN in cells transfected as in a. c, qPCR analysis of let-7a, let-7b, and let-7i in BE(2)C cells transfected for 2.5 days with control siRNA, siM1, or siM2 (n = 3 independent experiments, mean plus s.e.m. shown). d, Immunoblot analysis of indicated proteins in cells infected with indicated Cas9-gRNA lentivirus. For gel source data, see Supplementary Figures. e, Expression levels of let-7 targets in BE(2)C:MYCN cells transfected with siCon or siMYCN-3′ UTR. f, Relative let-7 expression in BE(2)C:MYCN cells co-transfected with siCon or siMYCN (3′ UTR) siRNA and miRCon or let-7a mimic. A 16-fold increase in let-7a results in an approximately eightfold increase in total let-7, owing to let-7a making up almost half of the total cellular pool (Fig. 3c, lower). g, Relative expression levels of let-7 targets in siCon and siMYCN cells transfected with let-7a mimic (data represent one round of mRNA-seq. ***P < 0.001, one-tailed Wilcoxon test, GSE81497, see Source Data F3).
Extended Data Figure 10 | Neuroblastoma patient and ENCODE data.

a. Detail of the incidence of chromosome 3p21 and 11q23 loss and MYCN amplification as determined by analysis of the indicated retrospective chromosomal aberration studies on neuroblastoma.

Extended Data Table 1 | Neuroblastoma patient and ENCODE data.

b. List of the ENCODE sRNA-seq samples analysed (with associated GEO accession numbers) for the relative expression of mature let-7 in Fig. 5c.

c. List of let-7 family host transcripts, transcript class, and let-7 location within the transcript.

d. List of the ENCODE mRNA-seq samples analysed (with associated University of California, Santa Cruz submission identifier numbers) for the relative expression of let-7 host transcripts in Fig. 5d.

e. Relative expression of let-7a2, let-7f2, and let-7g host genes by microarray in MYCN-amplified and non-amplified neuroblastoma. ACTB shown as control.*P < 0.05, **P < 0.01, ***P < 0.001, unpaired t-test, n = 643, Source Data (ED Fig 10).

f. Schematic showing the several mechanisms that impair let-7 biogenesis and function in neuroblastoma (chromosome images created at http://www.ncbi.nlm.nih.gov/genome/tools/gdp/).