Skoltech
Skolkovo Institute of Science and Technology

Thesis Changes Log

Name of Candidate: Anna Fefilova

PhD Program: Life Sciences

Title of Thesis: Functional study of human and murine Morrbid lncRNA *in vitro*

Supervisor: Prof Timofei Zatsepin

Chair of PhD defense Jury: Prof Yuri Kotelevtsev ***Email:*** *y.kotelevtsev@skoltech.ru*

Date of Thesis Defense: 15 December 2020

The thesis document includes the following changes in answer to the external review process.

I would like to thank all jury members for the careful review of my research work and valuable comments, which helped me make significant improvements to the thesis.

As a result of the reviews, I have made several changes reported in detail below.

Professor Pavel Ivanov

1. "Discussion: I would appreciate detailed discussion of different approaches for LOF studies of ncRNAs. It would be useful to add actual discussion on the limitations and benefits of specific approaches."

Modification to the thesis:

A section describing universally applied methods for LOF studies of lncRNA has been added in Discussion:

Most long non-coding RNA genes have not been studied yet and a complete picture of their regulatory features and the way they interact with each other and other molecules is yet to be revealed. One approach for the investigation of lncRNAs functions is loss-of-function methods, which specifically reduce the expression of a gene of interest and are powerful tools for the discovery of its biological functions. The LOF phenotype is obtained through the execution of gene silencing techniques either

involving genomic manipulations (such as CRISPR interference (CRISPRi), CRISPR/Cas9-mediated deletions, knock-ins, etc.) or targeted cleavage of an RNA transcript of interest (RNA interference or antisense oligonucleotides) [226]. A comparison of effectiveness and off-target effects of different LOF strategies demonstrated that each method has its limitations and weaknesses, and simultaneous application of several approaches is advised [211,227]. Unique biological features of lncRNAs must be considered when choosing a LOF method and interpretation the results. For example, lncRNA localization in some cases may influence the efficiency of the knockdown. Also, lncRNA may action in cis, in trans, or be non-functional. Nuclear localization and association with chromatin are indicators of cis-acting lncRNA regulator, cytoplasmic or nucleoplasmic lncRNAs most probably function in trans, while a possibility that a nuclear lncRNA is a non-functional by-product of transcription should also be considered. Additionally, regulatory DNA elements within the lncRNA locus or process of active transcription may both affect the expression of adjacent genes and lead to the phenotype that is unrelated to the lncRNA transcript itself.

An important advantage of knockdown techniques, which specifically degrade RNA molecule, while leaving lncRNA genomic locus and its transcription intact, is that they allow for the identification of lncRNA transcript-specific functions. RNA interference and antisense nucleotides are universally used for knockdown methods. RNAi utilizes transfected siRNAs molecules or endogenously expressed shRNA molecules to guide the RNA-induced silencing complex (RISC) to the target RNA molecule via complementary base pairing. Once a target lncRNA binds RISC-loaded siRNA/shRNA, it is cleaved by Ago2 and then degraded. A lot of evidence for lncRNA functional roles came from RNAi LOF studies. For example, the first affirmation that NEAT1 is a structural basis of paraspeckles came from siRNA-based phenotype assay in HeLa cells [228]. An efficient alternative to RNAi is provided by antisense oligonucleotides (ASOs), in which inhibitory activity is either promoted by catalytic RNaseH cleavage of the DNA:RNA substrates or blockage of pre-mRNA processing or translation by direct binding of modified oligonucleotides (steric-blocker oligonucleotides). It was demonstrated that RNaseH-dependent ASOs are more efficient in the nucleus than RNAi [229] and thus, for nuclear-bound lncRNAs ASOs represent a preferred knockdown method. Moreover, it was shown that ASOs lead to degradation of a nascent RNA transcript in the nucleus, thus mature lncRNA is never produced [230]. Locked nucleic acids (LNAs) are an important type of antisense oligonucleotides acting as steric blockers. LNAs are nucleic acid analogs, which are modified to have a ribose ring “locked” by a methylene bridge between the 2′ oxygen and the 4′ carbon. This modification results in a drastic increase of affinity to RNA and DNA. Xist targeting with LNAs resulted in its displacement from the inactive X chromosome and allowed for the identification of Repeat C as the essential for RNA localization at Xi [231]. Another steric blocking oligonucleotide approach is offered by antisense morpholino oligonucleotides (MOs), which are widely used to inhibit the expression of protein genes by preventing mRNA translation. MOs can also be

designed to bind lncRNA splice-sites and therefore deactivate RNA maturation or to target lncRNA functional regions, as it was done to investigate Cyrano and Megamind roles in zebrafish [156]. However, gene downregulation with synthetic nucleic acids possesses serious limitations. One of them is the risk of oversaturation of endogenous small RNA pathways, which may lead to abnormalities in cellular homeostasis, which can be mistaken for lncRNA dependent LOF phenotype [232]. Moreover, supraphysiological amounts of oligos may result in accumulation of aberrant RNA species inside the cell that can lead to non-specific changes in gene expression [233]. Minimization of these undesirable effects can be achieved through careful optimization of oligonucleotides sequences and doses before the transfection. Additionally, RNAi and ASOs techniques exhibit significant sequence-dependent off-target effects, that are extremely challenging to completely avoid [234,235]. One strategy, that was suggested to reduce off-target effects is targeting multiple regions within lncRNA transcript with a set of siRNAs/ASOs and pinpointing the concordance in phenotypes from individual probes [236]. Development of the novel methods targeting lncRNA transcripts for degradation continues. For example, the insertion of self-cleaving ribozymes into lncRNA sequences recently demonstrated promising results and proved to have efficiency similar to RNAi [237].

Various CRISPR-based methods have also been widely utilized in lncRNA studies. CRISPRi is a method that allows inhibition of lncRNA transcription without introducing changes into the underlying DNA sequence. CRISPRi uses a nuclease-deficient version of Cas9 (dCas9) which still possesses its RNA-dependent DNA-binding activity fused to the KRAB (Krüppel-associated box), which is recruited to the TSS of the target lncRNA by sgRNA and catalyzes repressive chromatin modifications around the TSS. CRISPRi has been successfully applied in high-throughput functional lncRNA characterization, such as a screen for growth regulators among more than 16 thousand lncRNAs in 7 different cell lines [238]

CRISPR/Cas9 is used to generate knockout of the target lncRNA via genomic excision of the entire lncRNA locus [239] or regulatory elements in the DNA sequence, like promoters [239]. Deletion of the entire gene does not allow to distinguish between lncRNA role versus DNA sequence. Also, depletion of such significant genome region may cause perturbations in the chromosome architecture, affect neighbor genes, resulting in phenotype changes. On the other hand, removal of just the promoter region terminates transcription, while leaving most of the DNA sequence unchanged. Another method Homology-Directed Repair (HDR) of the Cas9-induced DSBs (CRISPRn HR) can be used to knock-in DNA elements into lncRNA locus. Transcriptional terminator sequences inserted after TSS abolish the transcription while leaving the DNA sequence almost intact, providing a method, which allows distinguishing between the role of active DNA elements and the transcript/transcription [86].

CRISPR-based methods cannot be used to manipulate lncRNA loci which overlap PCGs, located antisense, expressed from a bidirectional promoter, or located close to a protein-coding gene. A genome-

wide analysis found that only 38% of all lncRNA can be safely genetically modified without the risk of disrupting the expression of neighbor genes [240]. For example, the CRISPRi approach was used to knockdown lncRNA NOP14-AS1 expressed from a bidirectional promoter of MFSD10 protein. All tested sgRNAs targeting NOP14-AS1 also lead to downregulation of MFSD10. However, NOP14-AS1 knockdown using antisense LNA GapmeRs did not affect NOP14-AS1 expression. A similar effect was obtained for 4 other lncRNA, including HOTAIR, as well as for protein-coding mRNA TP53 [240]. Like RNAi, CRISPR/Cas9 and CRISPRi technologies also exhibit off-target effects. Although they reported having higher fidelity [226], studies suggest off-target cannot be neglected when using CRISPR and dCas9 may bind up to 1000 off-target sites depending on sgRNA sequence [241]. Comparison between transcriptome profiles of the same lncRNA inhibited by RNAi, ASOs, or CRISPRi revealed little overlap in differentially expressed genes between these methods, suggesting significant method-specific off-target effects [226].

In this study, we chose to terminate transcription of hMorrbid and CYTOR by performing CRISPR/Cas9 excision of the promoter regions leaving the rest of the loci complete to avoid serious perturbations in the genome architecture. Also, we confirmed that deleted regions do not overlap any protein coding genes and located more than 2000 base pairs from the closest promoter as is recommended [240].

2. "Intro: NMD is a "nonsense-mediated mRNA decay" (and not 'nonsense-mediated decay'). I feel that this part should be explained in more details as it largely different from themes described in this thesis."

Modification to the thesis:

Corrected 'nonsense-mediated decay' to "nonsense-mediated mRNA decay" on pages 15, 142, 145, 164, 171.

A section describing NMD mechanism in more details was added to the Discussion:

Nonsense-mediated mRNA decay (NMD) is a protein synthesis quality control mechanism in cells that eliminates mRNAs containing premature termination codons (PTC) or other NMD-triggering factors. mRNA containing PTCs carry a potential threat to cell homeostasis since they can result in bulk production of non-functional proteins [265]. Detection of fault mRNAs occurs during the first round of mRNA translation. To discriminate between NMD-target and non-target mRNAs, cellular machinery utilizes either of two mechanisms: exon junction complex (EJC)-independent NMD and 3′ UTR EJC-dependent NMD, where the last is a more efficient process. All types of NMD pathways rely on RNA-dependent helicase and ATPase called UPF1 in addition to several other enzymes which can vary depending on a specific mechanism [266]. UPF1 binds to a single-stranded RNA disregarding the

sequence and uses ATP hydrolysis to move along the mRNA molecule in the 5'->3' direction. The core of the nuclear EJC is composed of eukaryotic initiation factor 4A3 (eIF4A3), which is a helicase that anchors the EJC to the RNA, RNA- binding protein 8A (RBM8A), and MAGOH52. This core is joined by other proteins, including UPF3X, which directly interacts with UPF2. EJC is positioned onto mRNA after it has been spliced in the nucleus ~20-24nt upstream of the exon-exon junction. During the first round of translation, the ribosome dislocates any protein complexes bound to mRNA, including EJC and UPF1. When translating ribosome encounters termination codon, the translation termination complex composed of eukaryotic release factor 1 (eRF1) and eRF3 is formed. UPF1 is recruited to eRF1–eRF3 translation termination complex together with the serine/ threonine kinase SMG1, forming an SMG1–UPF1–eRFs (SURF) complex. UPF3X in complex with UPF2 recruits UPF1 from the termination complex, bridging SURF to the EJC to form the decay- inducing (DECID) complex. This stimulates UPF1 helicase activity and promotes UPF1 phosphorylation by SMG1. UPF1 phosphorylation by SMG1 at multiple residues within its amino-terminal and carboxy-terminal regions is a commitment step in NMD. It prevents further rounds of translation initiation and is crucial for mRNA decay serving as a platform for recruitment of RNA degrading factors such as SMG5–SMG7 and SMG6. In EJC-dependent NMD, the EJC complex positioned downstream from the termination codon is required for stimulation of the NMD pathway. This feature justifies a '50–55 nucleotide rule': NMD occurs if a PTC located ≥50–55 nucleotides upstream of an exon-exon junction so that the leading edge of the terminating ribosome can't physically remove the EJC off the junction. On the other hand, the EJC-independent NMD pathway is activated in case mRNA contains longer than ~1 kb, unstructured 3′ untranslated region (3′ UTR), and mechanistically explained by the remote location of NMD inhibitor PABPC1 from the termination codon and therefore abolished initiation of a proper translation termination mechanism [265]. NMD is an essential modulator of gene expression, implicated in various physiological processes. It facilitates cellular response to environmental changes. Deregulation of NMD signaling in humans is associated with intellectual disability and cancer.

3. "Results and Discussion: I would add more information here with specific focus on why UPF1 is chosen for KD experiments to study the effects of mMorrbid in the regulation of NRAs mRNA isoforms."

Modification to the thesis:

The following description has been added to the Discussion:

To further verify NMD nature of NRAS-PTC cellular degradation, we silenced the central NMD factor UPF1 to simultaneously disrupt all circuitries leading to NMD activation in cells. Proteins UPF1, UPF2, UPF3 are well-studied key factors of the NMD pathway. While all three proteins were found

essential for NMD signaling in yeast cells, there have been several reports indicating that UPF2 and UPF3 can be dispensable in vertebrates under specific conditions and/or cell types. Several branches are leading to NMD activation and there is evidence that degradation of some PTC-containing mRNAs does not require UPF2 and UPF3 while all known NMD pathway converge at UPF1, making it a master regulator of NMD in vertebrates [266]. For example, in HeLa cells mRNP composed of Y14, MAGOH, and eIF4A3 was demonstrated to activate UPF1 phosphorylation independently of UPF2 and only a small subset of known NMD targets were upregulated in UPF3-depleted HeLa cells [268] UPF1 is essential for all NMD steps from PTC recognition to mRNA degradation.

4. "Also, it is not clear about the actual efficiency of UPF1 protein depletion in these experiments."

Modification to the thesis:

UPF1 western blot image was added to the Figure 39.

5. "It would be useful to hear why PhD candidate has chosen these or other cell lines as adequate models for such experiments. What are the alternative models that could be used in these studies?"

Response:

LncRNAs participate in the regulation of all known steps of gene expression and many of when have been implicated in illnesses, including hepatic diseases (doi:10.3390/ijms19030682). In the case of the identification of the therapeutically potent lncRNA in the liver, a rapid translation to animal models and clinical evaluation is possible due to unique liver physiology and recent progress in targeted drug delivery. LncRNAs can easily and specifically be targeted by RNAi and ASOs, which are powerful, safe, and cost-effective methods for gene silencing. ASO can be used for downregulation of nuclear lncRNAs and RNAi is efficient in degrading cytoplasmic lncRNAs. In the last years, many RNA based therapies underwent FDA approval, such as Patisiran (doi: 10.1038/s41565-019-0591-y), Givosiran (doi: 10.1007/s40265-020-01269-0) and many others with many more still investigated in clinical trials (doi: 10.1093/nar/gkw236). The main obstacle for the application of RNA therapies is the delivery of siRNA/ASOs into the cells of an organism, as several physiological barriers must be overcome first: degradation by nucleases in the serum, capture by phagocytic cells of the reticuloendothelial system, vascular endothelial wall barrier, clearance by the renal system (which filters molecules with sizes of 3-6nm a typical size for oligonucleotides) and the final challenge is targeted delivery of oligonucleotides to the specific organ or tissue. In this regard, the liver has been demonstrated as an excellent target with at least two efficient hepatocytes-specific delivery approaches: lipid nanoparticles (LNPs) and conjugates.

The most widely used and clinically advanced non-viral delivery approach is lipid nanoparticles (LNPs), complexes of anionic oligonucleotides with cationic lipids (doi: 10.1016/B978-0-12-800148-6.00004-3). LNPs have the size of approximately100nM, which in most of the tissues is too large to be transported across the vascular endothelial barrier, nevertheless, fenestrations between the endothelial cells in the liver, as well as in the spleen, allow egress of larger macromolecules and particles including LNPs into these tissues. Cationic and ionizable lipid-based nanoparticle formulations deliver

siRNA to the liver at more than 90% (doi: 10.1073/pnas.0910603106). Many siRNA-LNP complexes have been already tested in clinical trials (doi: 10.1016/B978-0-12-800148-6.00004-3), and majority developed to combat liver diseases: transthyretin-mediated amyloidosis, clotting disorders, liver cancer, and disorders of lipid metabolism (doi: 10.1038/nrg3978).

Another promising approach for drug delivery into the liver, which has already demonstrated its potency in the clinic, is a conjugation of oligonucleotides to a biomolecule capable of binding to a receptor present on the cells. A huge leap forward in the case of delivery to hepatocytes is attributed to the development of multivalent N-acetylgalactosamine (GalNac) conjugated siRNAs that bind at nanomolar levels to the asialoglycoprotein receptor (ASGR). ASGR is nearly ideal for the targeted delivery of oligonucleotides as it is predominantly displayed on the plasma membranes of hepatocytes at extremely high density (about 5 × 105 copies per hepatocyte) and is rapidly internalized and recycled with a turnover time of about 20 min. Several glycoconjugates-based therapies targeting hepatic genes have been progressing through clinical trials, including GalNac-siRNA conjugate for therapy of transthyretin-mediated amyloidotic cardiomyopathy, siRNA glycoconjugates addressing hemophilia A or B, antitrypsin deficiency, porphyria, and acute hepatic porphyria (doi: 10.1093/nar/gkw236, doi: 10.1007/s40265-020-01269-0).

Thus, liver availability for targeted RNAi/ASO-based drug delivery using LNPs or conjugates as vehicles makes it feasible to transition from in vitro to in vivo studies.

Our lncRNAs of interest Morrbid and CYTOR were reported to be upregulated in liver cancer. In addition to that, we found that expression of MIR-217 transcript, containing evolutionarily conserved region, was higher in hepatocytes cell lines HepG2 and Huh7 in comparison to other types of cells we tested (Figure 28B). This, together with the possibility to switch to translational studies in the future made us concentrate our attention on investigation of Morrbid function in hepatocytes. Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is the third leading cause of cancer-related deaths in the world with a limited amount of efficient treatment strategy developed (doi: 10.3390/ijms19030682). Several systemic agents have been developed and approved for the treatment of inoperable patients, like kinase inhibitors sorafenib and regorafenib, and immune checkpoint inhibitor nivolumab (doi: 10.3390/ijms19030682). However, the development of resistance towards current HCC

drugs is high, and overall patient survival is only prolonged by months, highlighting the need to search for new potential therapies and targets.

Hepatocellular carcinoma cell lines Huh7, HepG2, and Hep3B are relatively easy to maintain in culture and are widely used for studies. These cell lines preserved morphological features of hepatocytes, have robust reproducibility, widely available, relatively cheap, and allow for high-throughput studies (doi: 10.1016/j.dmpk.2018.03.003). Analysis of gene expression found differences between gene expression profiles between primary hepatocytes and hepatoma cell lines Huh7, HepG2, Hep3B. However, the same studies showed that cell lines greatly resemble each other (doi: 10.1016/j.dmpk.2018.03.003, doi: 10.1124/dmd.110.035873). Epigenetic studies suggested a more aggressive malignant phenotype of Hep3B compared to HepG2 or Huh7 cells (PMID: 18225533, doi:10.4236/jct.2013.42078). We chose to work with available in our laboratory Huh7 cell line. Huh-7 is a well-established and differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumor in a 57-year old Japanese male in 1982. Huh7 have been exploited to obtain detailed molecular information of multiple processes in liver malignant tumors, e.g. the biology of neoplastic hepatocytes; identification of tumor markers; efficacy of anti-cancer drugs and cellular responses; the progression of HCC to a metastatic state, etc. (doi:10.4236/jct.2013.42078).

6. "Regarding Fig. 31: Description in the text should be more clear. E.g., on page 126 it should be stated where transcript versus protein levels are measured (it is certainly about qRT-PCR based mRNA measurements, yet it sounds that for BMF, BAX and BAK, the levels of proteins were measured). It is also not clear to me why protein expression levels in WT, KO, M-217 and R-217 cells were not measured except for BCL-XL and MCL1."

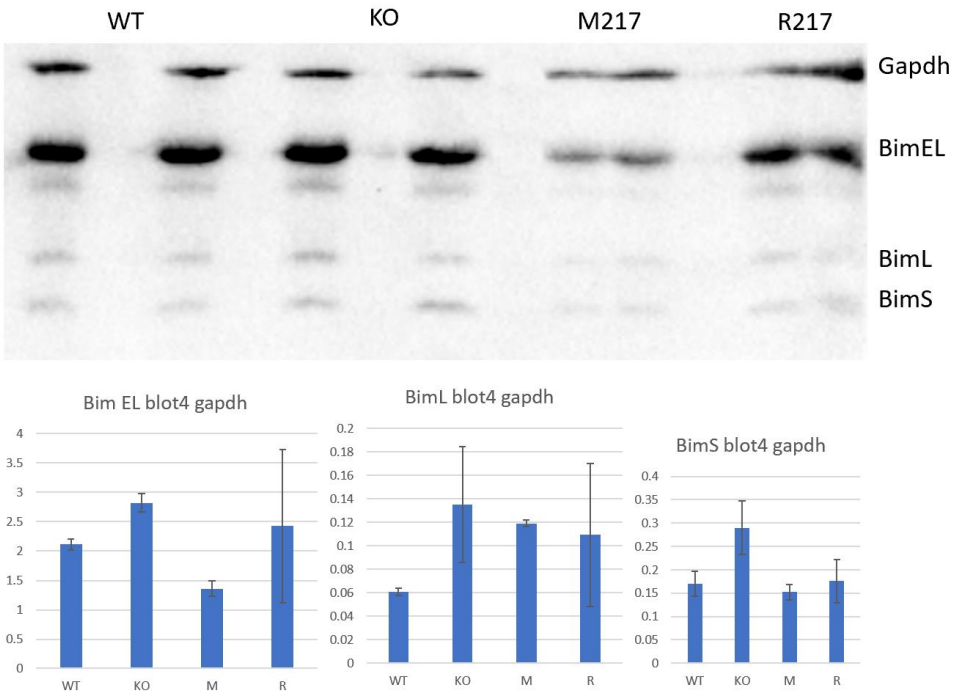
Modification to the thesis:

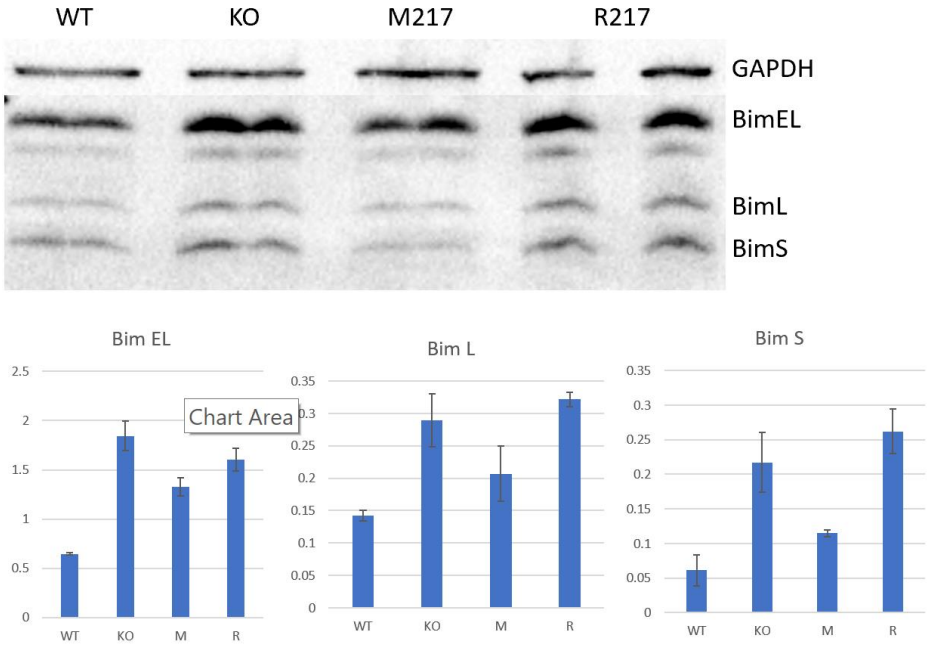
The author thanks reviewer for this valuable comment and in response to it RT-qPCR data was excluded from the Figure 31 as less relevant in this case, while western blot data for BAX and BAK pro-apoptosis proteins was added. Corresponding text modifications have also been made.

7. "Fig 32: It is difficult to judge the actual changes in the levels of BIM proteins. If possible, a more representative image of fig 32a could be shown."

Response:

Overall, I have repeated Bim protein measurement in WT, KO, M-217 and R-217 cell lines 5 times, at different times and cell passages. I find the image presented in the thesis the most representative, however, other western blot images I obtained (attached) also demonstrated slight upregulation of Bim isoforms in knockout cells and downregulation in M-217oe cells.





Professor Rory Johnson

8. "Nevertheless, some aspects of the story do not apparently lead to useful outcomes. One example of this is the finding that Morrbid regulates "poison-exon" splicing in NRAS. Apparently, this has no effect on mature NRAS mRNA or protein, so one struggles to

find the disease relevance here."

Response:

The author thanks the reviewer for this comment and agrees that even though regulatory role in NRAS alternative splicing was found for lncRNA Morrbid in hepatocytes, direct therapeutical relevance of this effect was not clear for the research model, which we studied. However, even in the case of protein-coding genes, LOF assays do not always lead to a particular phenotype clearly explaining the biological relevance of the gene (Helen Pearson. *Nature.* 2002. 415:8–9). In some cases, it can be due to parallel pathways compensating for one another or a redundant function shared by several genes. In other cases, phenotypes remain unidentified because the applied research method wasn't suitable for detection (Helen Pearson. *Nature.* 2002. 415:8–9). Oftentimes though, for the phenotype to become apparent, certain environmental conditions are required. For example, mice depleted in neuron-specific evolutionary conserved ncRNA BC1 displayed normal development, physiology, and lack of neurological defects or abnormal behavior in the lab environment (doi:10.1186/s13059-020-01994-5). However, when placed in natural outdoor conditions mutants failed to find distant food sources and had higher death rates than wild-type animals (doi:10.1186/s13059-020-01994-5).

Another reason for the lack of the obvious phenotype may be the wrong cell type, developmental stage, or other genetic factors. NEAT1 knockout mice lacked paraspeckles, however, remained viable and fertile (doi: 10.1083/jcb.201011110), suggesting that both NEAT1 and paraspeckles do not carry essential biological function. However, another group demonstrated that in luminal epithelial cells during mammary gland development silencing of NEAT1 results in abnormal mammary gland morphogenesis and lactation defects (doi: 10.1261/rna.047332.114). Moreover, later NEAT1 was found vital for phenotypic switching of vascular smooth muscle cells (doi: 10.1073/pnas.1803725115) and inflammatory responses in macrophages (doi: 10.1038/s41467-019-09482-6).

NMD mechanism besides being an mRNA quality-control mechanism is also exploited by cells to modulate transcriptome in response to environmental changes, like stress, differentiation, or development (doi: 10.1038/s41580-019-0126-2). It is not unlikely that in certain physiological or environmental conditions like stress, AS-NMD regulation of NRAS by Morrbid-SFPQ-NONO complex is required.

9. "Similarly, while Morrbid is an extremely complex locus, the choice to focus in on one single exon for deep study seems rather arbitrary (although admittedly, I don't have a better suggestion)"

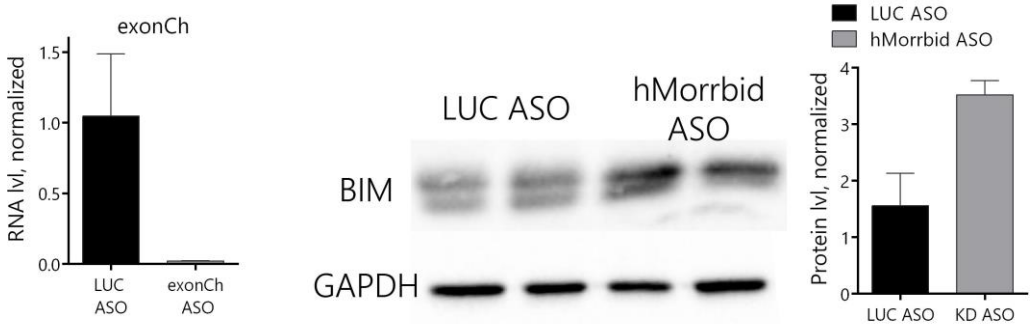
Response:

The reasons for choosing exonC are discussed in sections 2.7.2 ‘Morrbid sequence conservation between human and mouse’ on the page 64 and 4.5.1 ‘MIR4435-2HG (M-217) is a transcript of the hMorrbid gene containing evolutionary conserved region exonCh’ on the page 119.

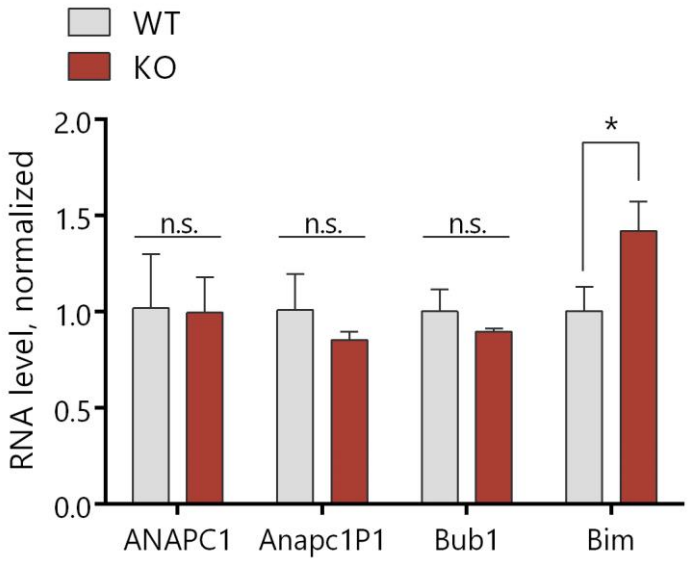
10. “Finally, the nature of the deletion in Morrbid cells was somewhat unclear to me – if a huge deletion between two chromosomal arms is occurring as implied in Figure 24, then there is the concern that observed phenotypes could result from other genomic elements in that region.”

Response:

The author agrees that ~4.5kbs of excised/mutated genomic sequence on the second chromosome could contain DNA regulatory elements, which potentially could influence the knockout phenotype. A particular concern represents genes located in the close genomic environment of the excision region, as they can be influenced by cis-acting DNA elements located within the lncRNA locus. We observed upregulation of adjacent to hMorrbid pro-apoptotic protein Bim in knockout cells (Figure 32). To verify that this effect is caused by inhibition of lncRNA transcript and is not related to DNA manipulations and/or hampered RNA transcription we also performed an ASO knockdown of exonCh. A mix of 4 ASOs targeting various regions of exonCh and individually tested was transfected into Huh7 cells for 24h and >90% knockdown efficiency was achieved. Then, we measured Bim protein level with western blot and found that it is significantly upregulated in exonCh knockdown cells in comparison to control knockdown. This result suggests that Bim expression is affected by the lncRNA transcript itself (and exonCh plays important role in it) and not by DNA elements within the deleted region.



Also, to confirm that CRISPR/Cas9 excision did not cause aberrant expression of neighbor genes due to perturbations in chromosomal architecture, we also measured the expression of genes located close to hMorrbid: ANAPC1, AnapcP1, Bub1, and Bim. That additionally demonstrated that in knockout cells, only Bim is upregulated in response to hMorrbid promoter deletion but no other proximal genes.



Professor Konstantin Lukyanov

11. "Page 110: "CYTOR is a sense lncRNA and hMorrbid is an antisense lncRNA." Please clarify what you mean for "sense" and "antisense" terms here. It is just opposite orientation of CYTOR and hMorrbid in the chromosome relatively to each other? Or is it a difference in their positions relatively to overlapping protein-coding genes in corresponding loci (as proposed in: Ma L, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. RNA Biol. 2013, 10, 925-933)?"

Response:

The term opposite orientation should be used here and was corrected in the thesis.

12. "Ref. 253: A misprint in the paper title ("eleme")."

Modification to the thesis:

Corrected

13. "observed delay" => "observed a delay " on p.4"

Modification to the thesis:

Corrected in the thesis

Professor Dmitri Pervouchine

14. "As a potential reader, I would be interested to know how transferable are the

findings on the regulation of murine NRAS splicing to its human ortholog and what implications it might have. The author doesn't discuss this matter until section 6.4, where the author says that "In human, no evidence for AS differential changes was found and the human NRAS gene does not produce PTC forms". I think it would be great to elaborate on how unusual it is, e.g., to give examples of human and murine lncRNA genes with diverged functions or to state it as a separate and important conclusion that she has identified the first such case"

Response:

The author finds this to be a valuable point and the following paragraph discussing the mentioned issue was added to the Discussion chapter.

Modification to the thesis:

In the case of protein-coding genes, it used to be generally be assumed that orthologous genes have the same functions [280]. However, research demonstrated that phenotypes associated with orthologous genes are oftentimes different between species [281]. One example is SPTLC2 gene, which disfunction causes non-identical physiological problems in human and in mouse [282]. Thus, even protein-coding orthologs can functionally diverge and an even more significant deviation can be expected from much faster evolving non-coding genes. A recent analysis of sequences and LOF transcriptomes of ortholog genes exhibiting phenotypic differences demonstrated correlation with changes in noncoding regulatory elements and tissue-specific expression profiles rather than changes in protein-coding sequences [282]. Cases, when functional repertoires of lncRNA orthologs with partial sequence conservation deviated throughout evolution, have been reported. For example, in human cells, HOTAIR lncRNA acquired novel functions in the regulation of HoxD locus, absent in mice [89]. Orthologs MEG3/Glt2 have been found to participate in a variety of non-overlapping cellular processes [166, 167]. Therefore, careful investigation of each case is required for a full understanding of lncRNA functional interspecies homology.

Professor Yuri Kotelevtsev

15. "In my opinion Discussion section is relatively short and could benefit from more detailed analysis of the own data in comparison with the previous findings of the function of described lncRNA in other cell types, particualy in leucocytes"

Response:

The author is thankful for this comment. Similarities and differences between Morrbid LOF phenotypes obtained in hepatocytes and in immune cells are discussed in the thesis, in particular influences on cell survival, apoptosis and regulation of Bim protein. I am also looking forward to

discussions during the thesis defense.