WISP1-AS1, a long noncoding RNA, upregulated in human renal cells exposed to the mycotoxin ochratoxin A and in human renal cancer cells

Dissertation

for obtaining the

Doctoral degree in natural sciences (Dr. rer. nat.)

at the

Faculty of Natural Sciences I – Biosciences –

Martin Luther University

Halle-Wittenberg

submitted by

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Opening of the doctoral procedure on 19.12.2019
Doctoral dissertation was publicly defended on 31.07.2020

“Die Schwerste aller Sprachen ist scheinbar Klartext.”
“The hardest of all languages seems to be clear text.”

Abstract

Introduction: Ochratoxin A (OTA) is a worldwide spread mycotoxin that contaminates our food. Detrimental effects of OTA on human health are evident by damage to renal cells, causing nephropathies. Long non-coding RNAs (lncRNAs) are also implicated in the pathogenesis of renal nephropathies and cancers. Herein I report on a novel lncRNA, *WISP1-AS1*, an antisense transcript in the *WISP1* locus. OTA is the only known inducer of *WISP1-AS1*. However, *WISP1-AS1* functions in OTA-treated cells were undiscovered. This research was set to investigate *WISP1-AS1* characteristics and functions in OTA toxicity and renal cancer cells and to understand the molecular mechanisms behind it.

Methods: *WISP1-AS1* expression in normal and cancer renal cells was investigated by PCR methods and Northern blot. *WISP1-AS1* knockdown was established by LNA™ GapmeR antisense oligonucleotides (ASOs). By RNA-sequencing, influence of *WISP1-AS1* on transcriptome was evaluated. Changes in transcription factor (TF) activities were analyzed by promoter activity assays. Glucose consumption and lactate production were determined by specific enzymatic assays. The effect of *WISP1-AS1* on cell death (apoptosis and necrosis) was assessed by measuring caspase activities and lactate dehydrogenase (LDH) release. Nuclear fragmentation and mitochondrial membrane potential were analyzed by Cytation 3 Cell Imaging Multi-Mode Reader.

Results: *WISP1-AS1* transcribes in antisense in comparison with *WISP1* mRNA. Expression in antisense was confirmed by Northern blot. *WISP1-AS1* transcription is completely inside the *WISP1* locus and does not suppress *WISP1* mRNA. *WISP1-AS1* influences the activity of certain TFs. Promoter activity assays confirmed that the activity of TF EGR-1 was increased and E2F activity was suppressed in a *WISP1-AS1*-dependent manner. *WISP1-AS1* expression was elevated in renal cancer cells and OTA further upregulated it. High *WISP1-AS1* expression in renal cancer cells without OTA treatment indicates its implication in carcinogenesis. Functional experiments demonstrate that *WISP1-AS1* might direct glucose towards mitochondrial respiration to obtain ATP. In *WISP1-AS1* presence, caspase-3 activity was significantly low and the *GAS6* gene, involved in caspase-3 suppression, was upregulated.

Conclusions: *WISP1-AS1* is an antisense noncoding transcript. *WISP1-AS1* upregulates the activity of TF EGR-1 and suppresses the activity of E2F. It lowers caspase-3 activity and acts antiapoptotic, possibly by upregulating *GAS6*. In *WISP1-AS1* presence, OTA-treated cells maintain normal energy metabolism.

Keywords: lncRNA, antisense, WISP1, ochratoxin A, antiapoptotic, renal cell

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1 Introduction

1.1 Focus on regulatory noncoding RNA molecules

Between 70–90 % of base pairs (bp) in our genome will be transcribed in some cell types at some point during our lives [Carninci et al. 2005; E. P. Consortium 2012; E. P. Consortium et al. 2007; Djebali et al. 2012]. However, only around 2 % of those bp transcribes into protein-coding messenger RNA (mRNA) [E. P. Consortium 2012; I. H. G. S. Consortium 2004], included in the databases such as GENCODE and Ensembl [Harrow et al. 2012; Hubbard et al. 2002]. The rest of transcripts comprise a diverse class of noncoding RNAs (ncRNAs), including infrastructural or housekeeping RNAs and regulatory RNAs (Fig. 1.1) [reviewed by Ponting et al. 2009].

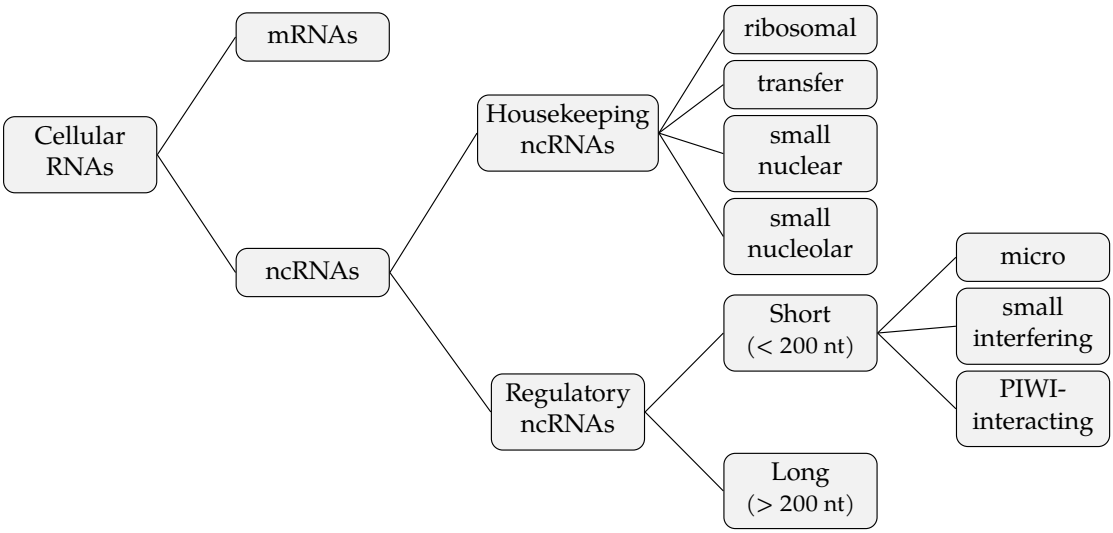


Figure 1.1: Cellular RNA classification. Long ncRNAs are subdivided in Fig. 1.2.

NcRNAs function as RNA molecules without translation into proteins. According to the central dogma [Crick 1970], RNA was only thought to be a messenger between DNA and proteins. However, their involvement in gene expression is larger than initially thought [Lozada-Chávez et al. 2011 and Fig. 2 there within; Marques et al. 2014 and Fig. 2 there within]. This ncRNA awareness, started in the 2000s in the postgenomic era[1], caused a sharp genome-wide increase in discovered regulatory ncRNAs [Bertone et al. 2004; Imanishi et al. 2004; Kampa et al. 2004].

Noncoding transcripts are functionally important. They contain "much of the information required to program our development" [Mattick 2010] and are involved in all functional aspects within the cell in health and disease [reviewed

[1] After sequencing of the human genome; https://www.genome.gov/human-genome-project

by ESTELLER 2011; MATTICK 2001]. The size of the noncoding genome scales with the complexity of the organism – unlike the protein-coding genome [TAFT ET AL. 2007; reviewed by HO 2014; MATTICK 2010; PRASANTH AND SPECTOR 2007]. Protein-coding genes alone are inadequate to justify the complexity of higher eukaryotic organisms [reviewed by SZYMANSKI AND BARCISZEWSKI 2002]. NcRNAs are filling the gaps in our understanding of the cellular functions.

For this reason, currently one of the most important challenges is to understand the functions of regulatory ncRNAs and the conditions of their expression in human diseases.

1.2 Recognized importance of long noncoding RNAs

Long noncoding RNAs (lncRNAs) are a class of ncRNAs longer than 200 nucleotides (nt). This limit was chosen to separate them from other noncoding and regulatory RNAs, which are typically shorter than 200 nt. Mattick and J. L. Rinn believe the lncRNA term and its classification by length and noncoding feature will be used only temporarily "until they are better characterized" [2015].

The first lncRNA with a described function was *H19* [BRANNAN ET AL. 1990], soon followed by the second, X-inactive specific transcript (*XIST*) [BROCKDORFF ET AL. 1992; BROWN, BALLABIO, ET AL. 1991; BROWN, HENDRICH, ET AL. 1992]. Both lncRNAs are involved in epigenetic regulation.

Before lncRNA functions were investigated, it was thought that such transcripts have no function in the cell and were defined merely as "transcriptional noise" [BLAKE ET AL. 2003] or the "dark matter in the genome" [J. M. JOHNSON ET AL. 2005; KAPRANOV ET AL. 2010]. These conclusions were reached due to the lack of evidence of function and the inappropriate focus on the characteristics valid for the coding sequences (e.g. protein-coding, sequence conservation across species and high expression) [discussed in PONTING ET AL. 2009].

Thus, it is no wonder that pervasive genome transcription stayed unappreciated until the use of whole-genome technologies. Particularly, importance of lncRNAs was recognized through microarrays and sequencing experiments [BERTONE ET AL. 2004; CHENG ET AL. 2005; MERCER ET AL. 2011; L. YANG ET AL. 2011].

To date[2], 96 308 genes for lncRNAs have been identified in humans, transcribing into 172 216 lncRNAs, according to the most comprehensive lncRNA annotation database, the NONCODE database [Y. ZHAO, H. LI, ET AL. 2016]. Another database, LNCipedia [VOLDERS ET AL. 2013] reports 56 946 lncRNA

[2] 30 November 2019

genes and 127 802 lncRNA transcripts. Many lncRNAs discovered by whole-genome sequencing experiments have only *in silico* predicted functions [PERRON ET AL. 2017; reviewed by CAO ET AL. 2018; SIGNAL ET AL. 2016]. While annotations are being improved [FRANKISH ET AL. 2018; SZCZEŚNIAK ET AL. 2019; reviewed by USZCZYNSKA-RATAJCZAK ET AL. 2018], most lncRNAs still lack experimental verification of their predicted functions.

In the last few years, astounding effort was put into experimentally revealing molecular characteristics and functions of lncRNAs, mostly in the context of human diseases [BLONDEAU ET AL. 2015; YAN ET AL. 2015; YUAN ET AL. 2016]. Finding functions is possible with many new technologies [reviewed by BESTER ET AL. 2018; JATHAR ET AL. 2017; KASHI ET AL. 2016; QIAN ET AL. 2019]. Experimentally confirmed and published functions of lncRNAs are curated into a reference database, the lncRNAdb [QUEK ET AL. 2015]. Thus far it counts 290 lncRNAs.

LncRNAs are implicated in regulation of possibly all cellular (patho)physiological processes, from development, metabolism, aging to diseases [reviewed by BATISTA AND CHANG 2013; GEISLER AND COLLER 2013; SOUSA-FRANCO ET AL. 2019; X.-Y. ZHAO AND LIN 2015]. Therefore, research of their functions is an important link in deciphering the cellular molecular functions in health and disease.

1.3 Similarities between coding and long noncoding RNAs

Coding and long noncoding RNAs share many molecular characteristics. Both utilize the same transcriptional machinery, including RNA polymerase II (RNA Pol II) [GUTTMAN ET AL. 2009]. Their promoters are occupied by RNA Pol II and certain histone modifications [CONLEY AND JORDAN 2012; SATI ET AL. 2012]. The majority has a 5′ cap and 3′ poly(A) tail [GUTTMAN ET AL. 2009; SIGOVA ET AL. 2013]. They show similar regulation by transcription factors (TFs) and number of identical TF binding sites (TFBSs) in the promoters [CAWLEY ET AL. 2004].

Similar to mRNA, "more than 25 % of lncRNA genes show evidence of alternative splicing with at least two different transcript isoforms per gene locus" [CABILI, TRAPNELL, ET AL. 2011; DERRIEN ET AL. 2012]. In both articles, the authors also observed that lncRNAs tend to have only two exons, which makes them shorter transcripts than mRNAs. Other researchers found that "nearly every noncoding exon is subject to alternative splicing" [DEVESON ET AL. 2018].

Another shared feature are open reading frames (ORFs). Protein-coding ORFs are usually conserved [SIEPEL ET AL. 2005]. LncRNAs either have conserved ORFs [CHEKULAEVA AND RAJEWSKY 2019] or non-conserved ORFs, the latter appearing by chance during evolution [DINGER ET AL. 2011]. To discriminate coding

from noncoding RNA only by ORF features becomes complicated for lncRNAs originating from pseudogenes, like *XIST* whose ORFs are conserved, but have lost their protein coding role [ELISAPHENKO ET AL. 2008; reviewed by HEZRONI ET AL. 2017; SHEVCHENKO ET AL. 2013].

There is evidence that some lncRNAs code for a peptide with biological function [ANDREWS AND ROTHNAGEL 2014; JACKSON ET AL. 2018; RAZOOKY ET AL. 2017], although many computationally predicted ORFs are not yet investigated for transcription and functionality [CALVIELLO ET AL. 2015; JI ET AL. 2015]. Additionally, evidences of dual coding and noncoding RNAs were found [HUBÉ AND FRANCASTEL 2018; NAM ET AL. 2016].

1.4 Specific lncRNA features

LncRNAs are less sequence conserved than protein-coding genes [reviewed by FANG AND FULLWOOD 2016], because of two reasons. First, their sequences evolve fast within placental mammals/primates [DERRIEN ET AL. 2012; NECSULEA ET AL. 2014]. Second, their secondary structural elements and tertiary domains are both conserved and important for the function [DELLI PONTI ET AL. 2018; reviewed by BLYTHE ET AL. 2015; QIAN ET AL. 2018]. Without sequence homology, human *Alu* and mouse *Sineb2* (short interspersed elements B2) RNAs fold into similar structures and bind RNA Pol II [ESPINOZA ET AL. 2004; MARINER ET AL. 2008]. Despite low sequence conservation, promoter regions of lncRNAs are highly conserved [PONJAVIC ET AL. 2007], equal to or more than the promoters of protein-coding transcripts [CARNINCI ET AL. 2005; DERRIEN ET AL. 2012].

LncRNAs are less abundant than mRNAs, meaning they are expressed at significantly lower levels [DERRIEN ET AL. 2012; Table 1 in PALAZZO AND LEE 2015]. Djebali et al. estimated that only a low number of lncRNAs (< 1000) appear in more than one copy per cell (in common human cell lines) [2012]. Some transcripts are expressed as low as 0.0006 copies per cell [MERCER ET AL. 2011]. This makes it harder to find their function unless certain cells and conditions are tested [PALAZZO AND LEE 2015]. The low amount is explained by their function as regulatory molecules [reviewed by MATTICK AND MAKUNIN 2006]. Exceptions are some highly abundant structural lncRNAs, important for the maintenance of chromatin and nuclear architecture, [reviewed by MELÉ AND J. RINN 2016] for example *NEAT1*[3] [CLEMSON ET AL. 2009; reviewed by LO ET AL. 2016].

The expression of a lncRNA is usually spatially restricted to certain tissues or cell types [CABILI, TRAPNELL, ET AL. 2011; DERRIEN ET AL. 2012; JIANG ET AL. 2016;

[3] nuclear paraspeckle assembly transcript 1

reviewed by WARD ET AL. 2015] and tightly timely regulated [PETRI ET AL. 2015; X.-Q. ZHANG ET AL. 2017]. For example, while lncRNA *FENDRR*[4] expresses only in mesoderm during normal development [GROTE ET AL. 2013], many other lncRNAs are specifically enriched in testes [CABILI, TRAPNELL, ET AL. 2011; NECSULEA ET AL. 2014]. With tight spatio-temporal control, lncRNAs possess a potential to finely control gene expression [BATISTA AND CHANG 2013], although it makes them harder to discover [DJEBALI ET AL. 2012; KASHI ET AL. 2016].

LncRNAs predominantly accumulate in the nucleus [DERRIEN ET AL. 2012; DJEBALI ET AL. 2012; GUDENAS AND L. WANG 2018], associating with the chromatin or specific nuclear substructures [DERRIEN ET AL. 2012; DJEBALI ET AL. 2012; reviewed by GUDENAS AND L. WANG 2018; MAGISTRI ET AL. 2012]. Nuclear localization supports their functionality without translation due to compartmentalization away from the translational machinery [CABILI, DUNAGIN, ET AL. 2015; JI ET AL. 2015]. This localization provides valuable clues to understand their molecular functions [reviewed by CABILI, DUNAGIN, ET AL. 2015; L.-L. CHEN 2016]. However, some lncRNAs are cytoplasmic [L.-L. CHEN 2016; reviewed by NOH ET AL. 2018], cytoplasmic and nuclear [CABILI, DUNAGIN, ET AL. 2015], or shuttle between the compartments, including mitochondria, upon stimulation [CARRIERI, FORREST, ET AL. 2015; KINO ET AL. 2010; reviewed by NOH ET AL. 2018].

1.4.1 LncRNA orientation and genomic location

To define the origin from the genome, two characteristics are important: orientation and location. Orientation is defined in respect of the nearest protein-coding gene: sense (in the same direction) or antisense (in the opposite direction) [DERRIEN ET AL. 2012; reviewed by J. L. RINN AND CHANG 2012].

The location can be either in the genomic space between protein-coding genes, so called gene desserts (*inter*genic lncRNAs) or in the parts of the protein-coding gene (*intra*genic lncRNAs) [DERRIEN ET AL. 2012]. Intergenic lncRNAs are called long intergenic noncoding RNAs (lincRNAs) [reviewed by PONTING ET AL. 2009; RANSOHOFF ET AL. 2018]. Intragenic lncRNAs can be transcribed from the sense or antisense strand, each either entirely from intron (intronic sense or intronic antisense lncRNA) or overlap parts of exon [overlapping sense lncRNA or natural antisense transcript (NAT)].

Bidirectional lncRNAs are a separate class. They transcribe from a close proximity (< 1000 bp) of the protein-coding gene and always in the opposite (divergent) orientation. In contrast to bidirectional lncRNAs, lincRNAs are located far

[4] Forkhead box protein F1 adjacent non-coding developmental regulatory RNA

from the protein-coding genes.

LncRNA classes are presented in Fig. 1.2 and reviewed by B. HRDLICKOVA ET AL. 2014; JARROUX ET AL. 2017; KAZEMZADEH ET AL. 2015.

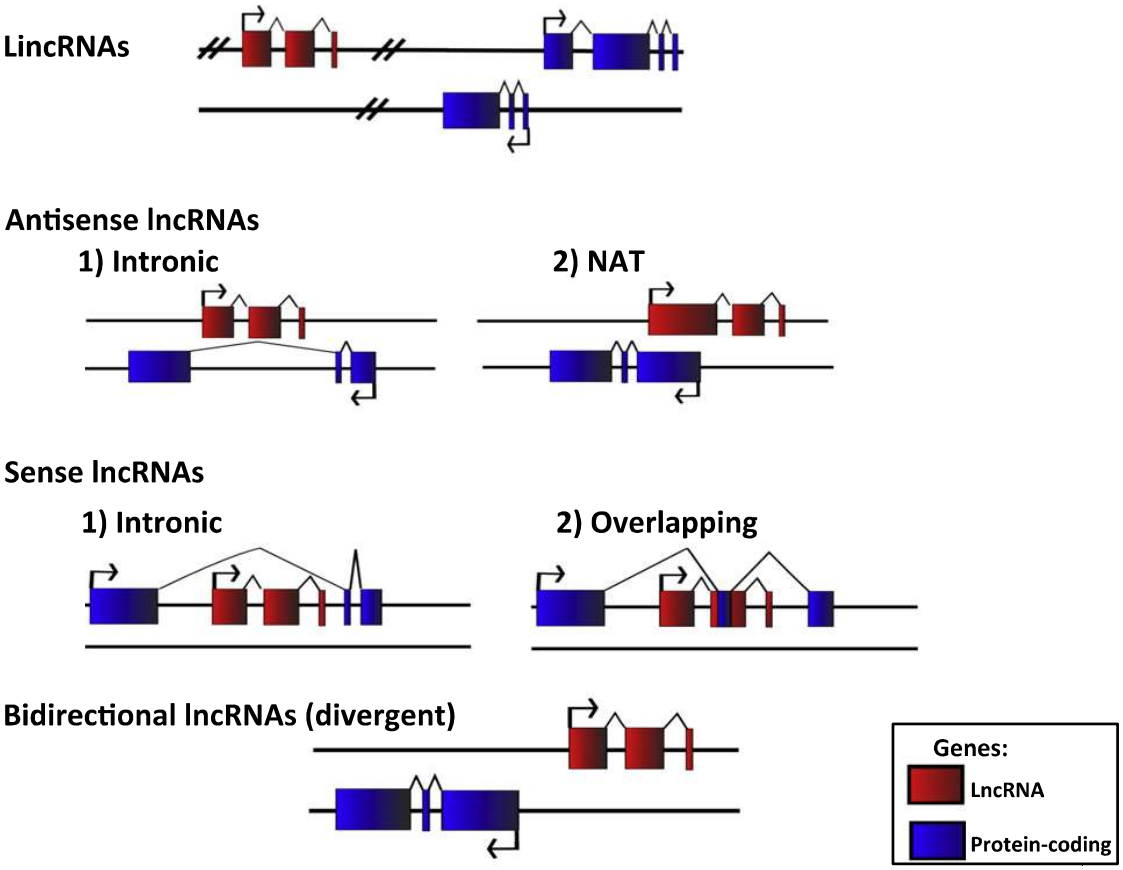


Figure 1.2: Long noncoding RNA classes based on their genomic location with respect to protein-coding genes. Long intergenic noncoding RNAs (lincRNAs) are transcribed from the region between two genes. Antisense lncRNAs overlap in antisense the introns or at least a part of the exons of the sense gene. Sense lncRNAs overlap in sense the introns or exons of the sense gene. Bidirectional lncRNAs transcribe in the opposite direction and from the vicinity of the sense gene. Image taken from B. HRDLICKOVA ET AL. 2014. Copyright 2019, with permission from Elsevier.

1.5 Natural antisense transcripts (NATs)

Natural antisense transcripts (NATs) are endogenous, processed transcripts containing partial or complete complementarity with known sense transcripts. Sense and antisense transcripts can be either coding or noncoding but usually NATs are noncoding RNAs, antisense to a protein-coding sense RNA [FAGHIHI AND WAHLESTEDT 2009; KATAYAMA ET AL. 2005]. Throughout my thesis I will focus on long noncoding NATs. Long noncoding NATs are mainly localized in the nucleus, mostly enriched in chromatin [DERRIEN ET AL. 2012] and appear

highly tissue-specific (analyzed in various tissues and species) [reviewed by VANHÉE-BROSSOLLET AND VAQUERO 1998; WERNER ET AL. 2007].

NATs are transcribed and act *in cis* or *in trans*. *Cis*-NAT represents a transcript from the opposite strand but the same genomic locus compared with its target sense transcript. Due to their sequence complementarity they present "a perfect pair". *Trans*-NAT, on the other hand, is transcribed from a distinct genomic locus compared with its target sense transcripts. They require only partial sequence complementarity and hybridize with some mismatches [reviewed by KOPP AND MENDELL 2018; VANHÉE-BROSSOLLET AND VAQUERO 1998].

Antisense transcription is more pervasive than the previously thought 22–47 % in a polyadenylated fraction [J. CHEN ET AL. 2004; ENGSTRÖM ET AL. 2006; OZSOLAK ET AL. 2010]. Katayama et al. discovered formation of sense-antisense pairs in 70 % of all human and mouse genomic loci, which could indicate functionality [2005]. NATs are usually 10-fold less abundant than corresponding sense expression [HE ET AL. 2008] and the reduction can be even 1000-fold [WENRIC ET AL. 2017]. There are exceptions, e.g., *BACE1-AS* is more abundant than *BACE1*[5] mRNA in brain of the patients with Alzheimer's disease [FAGHIHI, MODARRESI, ET AL. 2008] and the overall expression of NATs in breast tumors is increased in comparison with sense transcripts [WENRIC ET AL. 2017].

1.5.1 Mechanisms of NAT action

NATs have two general mechanisms of action on their coding counterparts: concordant and discordant. In concordant (positive) regulation, NAT stimulates expression of the target gene or NAT expression stabilizes the target sequence. Discordant (negative) regulation refers to repressive action of NAT on the target gene; when NAT is increased, expression of target gene is inhibited. The two mechanisms are graphically presented in Fig.1 in WIGHT AND WERNER 2013.

K. C. Wang and Chang introduced four archetypes that summarize lncRNA functional mechanisms and that apply to NATs [2011]. To show the diversity of NAT's involvement in cellular functions, I categorized NAT mechanisms observed in humans according to the level at which NAT influences gene expression regulation and the type of functional interaction (Table 1.1). The structure was adopted from FAGHIHI AND WAHLESTEDT 2009; VILLEGAS AND ZAPHIROPOULOS 2015; reviewed by PELECHANO AND STEINMETZ 2013. Nuclear interactions are reviewed by MARCHESE AND RAIMONDI 2017.

[5] beta-site amyloid precursor protein cleaving enzyme 1 also known as beta-secretase 1

Table 1.1: The mechanisms of natural antisense transcripts

Level of gene regulation	Type of functional interaction	Section
Pretranscriptional	NAT-chromatin-modifiers (guide, signal and scaffold archetypes)	1.5.1.1
	NAT-DNA	1.5.1.2
Transcriptional	Transcriptional interference	1.5.1.3
Posttranscriptional	NAT-RNA in the nucleus	1.5.1.4
	NAT-RNA in the cytoplasm	1.5.1.5
Translational	Translational interference	1.5.1.6
Posttranslational	NAT-protein (decoy archetype)	1.5.1.7

1.5.1.1 NAT-chromatin-modifiers interactions

NATs can interact with DNA- and histone-modifying enzymes [reviewed by MAGISTRI ET AL. 2012] and induce epigenetic changes in gene activity [reviewed by ALLIS AND JENUWEIN 2016]. Basically, they can activate gene expression by interacting with enzymes that either remove repressive DNA marks or add active histone marks. To silence gene expression, NATs interact with enzymes that add repressive DNA and histone marks (Fig. 1.3). Influences of NATs on epigenetic modifications are explained through the following archetypes.

Guide archetype

NATs can recruit regulatory proteins to the target gene locus either *in cis* or *in trans*. Guidance *in cis* occurs cotranscriptionally [K. C. WANG AND CHANG 2011]. Guidance *in trans* [reviewed by HUNG AND CHANG 2010] is realized by RNA:DNA heteroduplex or RNA secondary structures. Interaction between NAT and DNA is direct or mediated by another protein (Fig. 1.4) [B. HRDLICKOVA ET AL. 2014].

To repress gene expression, guide NATs bind DNA methylation enzymes, DNA methyltransferases (DNMT), and recruit them to the promoters of NAT's target genes [Y. ZHAO AND H. SUN H. W. 2016]. DNA methylation is involved in induction of inactive chromatin and stalls gene expression [reviewed by GUJAR ET AL. 2019; MIRANDA AND JONES 2007]. *DUM*[6], an antisense transcript to the developmental pluripotency-associated protein 2 (*DPPA2*) gene interacts with DNMTs and recruits them to the *DPPA2* promoter. Thus, *DUM* silences *DPPA2 in cis* and promotes differentiation of skeletal muscle cells [L. WANG ET AL. 2015].

Scaffold archetype

NAT presents a platform to which diverse protein complexes attach and get directed to a certain genomic location. NAT forms unique secondary structures

[6] developmental pluripotency-associated 2-upstream binding muscle lncRNA

which can be recognized by different proteins. Single NAT can bind multiple proteins concurrently and bring regulatory factors (transcription activators and repressors) in the vicinity to perform complex acts in gene expression regulation (Fig. 1.4) [K. C. WANG AND CHANG 2011].

To repress gene expression, scaffold NATs bind histone-modifying enzymes [reviewed by MAGISTRI ET AL. 2012]. An example is HOX transcript antisense RNA (*HOTAIR*) transcribed from the *HOXC* locus [J. L. RINN, KERTESZ, ET AL. 2007]. *HOTAIR* simultaneously binds two chromatin-modifying complexes: one that introduces repressive histone mark and another that removes active histone mark. Both protein complexes work toward silencing gene expression *in trans* over 40 kilobases (kb) of the *HOXD* locus [J. L. RINN, KERTESZ, ET AL. 2007].

To activate gene expression, NATs induce DNA demethylation. Demethylation can be passive (lost through cell divisions) or active (mediated by a multistep enzymatic process) [reviewed by BOCHTLER ET AL. 2017; WU AND Y. ZHANG 2014]. *TARID*[7], an antisense transcript to the transcription factor 21 (TCF21), physically binds *TCF21* promoter and a GADD45A[8] protein that recruits an enzyme to remove methylated cytosine. Therefore, *TARID* serves as a scaffold and a guide for demethylating machinery [ARAB ET AL. 2014].

NAT can activate gene expression by interacting with chromatin modifiers. HOXA transcript at the distal tip (*HOTTIP*) is transcribed from the 5′ of the *HOXA* locus. *HOTTIP* binds a part of the histone methyltransferase complex and guide the complex to the *HOXA* locus. The complex adds active histone marks and activates transcription of multiple genes across 40 kb [K. C. WANG, Y. W. YANG, ET AL. 2011].

Signal archetype

NAT transcribes at a certain place and time, thus, NAT is considered a molecular signal, marking the spatio-temporal event, e.g., developmental stage or respond to stimuli. Just the act of transcription is regulatory but NATs can have additional functions (Fig. 1.4) [K. C. WANG AND CHANG 2011].

X-chromosome inactivation is the mechanism to compensate for the dose of X-chromosome-linked genes in XX and XY individuals. Inactivation of one entire X chromosome in females is controlled by transcription of a NAT, *XIST* [BROCKDORFF ET AL. 1992; BROWN, BALLABIO, ET AL. 1991; BROWN, HENDRICH, ET AL. 1992]. *XIST* spreads from X inactivation center (*XIC*) along the X chromosome and triggers heterochromatin formation by acting as a guide for the chromatin

[7] transcription factor 21 antisense RNA-inducing demethylation
[8] growth arrest and DNA-damage-inducible 45 alpha

remodeling protein PRC2[9]. *XIST* initiates silencing *in cis* [C CHANG ET AL. 2006; J. ZHAO ET AL. 2008] and marks the inactive X chromosome.

1.5.1.2 NAT interactions directly with DNA

Antisense transcripts can form RNA:DNA hybrids or triplex structures with genomic DNA. In these structures, NAT can additionally recruit epigenetic modifiers and change the gene status [reviewed by Y. LI ET AL. 2016].

ANRASSF1[10] forms RNA:DNA hybrid with the *RASSF1A*[11] promoter, then binds and recruits PRC2 at the *RASSF1A* promoter. PRC2, in turn, induces histone methylation and *RASSF1A* downregulation [BECKEDORFF ET AL. 2013]. Another example is promoter-associated RNA (*pRNA*), antisense to the promoter of ribosomal DNA (*rDNA*) clusters. This NAT binds to *rDNA* and forms RNA:DNA triplex, specifically recognized by DNMT3b which methylates *rDNA* promoter and silences *rDNA* genes [SCHMITZ ET AL. 2010].

1.5.1.3 Transcriptional interference

Instead of RNA product, *the act of NAT transcription* can regulate gene expression *in cis*. Transcriptional interference can occur during the transcriptional initiation or elongation phase between two convergent transcriptional units which overlap on the opposite strands. For example, two convergent promoters can compete for RNA Pol II in the transcription initiation. Likewise, RNA Pol II can physically collide with the complex from the opposite strand; transcription then stops from one or both strands (Fig. 1.3) [SHEARWIN ET AL. 2005].

Transcription of *GNG12-AS1*[12] interfere with a neighboring tumor suppressor. The transcription start site (TSS) of *DIRAS3* and *GNG12-AS1* compete for the binding of RNA Pol II. Resulting *DIRAS3* transcription is only possible only when *GNG12-AS1* transcription is stalled [STOJIC ET AL. 2016].

Protein-coding gene *IGF2R*[13], imprinted on the maternal allele, [BARLOW, STÖGER, ET AL. 1991] contains a convergent cis-acting NAT, *AIRN*[14] [YOTOVA ET AL. 2008; ZWART ET AL. 2001]. An imprint is DNA methylation on the imprinting control element (ICE) [reviewed by BARLOW AND BARTOLOMEI 2014; BARTOLOMEI AND FERGUSON-SMITH 2011], located at a CpG island within an intron of the

[9] polycomb repressive complex 2
[10] antisense to Ras association domain family member 1
[11] Ras association domain family member 1
[12] G protein subunit gamma 12-antisense RNA 1
[13] insulin-like growth factor type 2 receptor
[14] antisense to insulin-like growth factor type 2 receptor RNA noncoding

IGF2R gene [STÖGER ET AL. 1993]. Methylated ICE prevents *AIRN* transcription and, in turn, allows *IGF2R* transcription. On the paternally inherited allele, ICE is nonmethylated, allowing *AIRN* to transcribe and, thus, prevent the transcription of *IGF2R* [KOERNER ET AL. 2009; LATOS ET AL. 2012; SLEUTELS ET AL. 2002]. *AIRN* transcription, but not the molecule itself, is important for the silencing of *IGF2R* gene [LATOS ET AL. 2012; SEIDL ET AL. 2006].

1.5.1.4 NAT-RNA interactions in the nucleus

RNA masking

When an antisense transcript binds to sense mRNA, it forms double-stranded RNA (dsRNA). This physically prevents the binding of regulatory factors to mRNA part in a duplex.

NAT can promote the translation of mRNA by masking splice sites (Fig. 1.3). *ZEB2-AS1*[15], upregulated during epithelial-mesenchymal transition (EMT), binds to the 5′ of *ZEB2* pre-mRNA and covers the splice site. Hence, the first intron with an element for efficient *ZEB2* translation remains in the splice variant and enables translation [BELTRAN ET AL. 2008; Fig. 5 in SERVISS ET AL. 2014]. Likewise, NATs hold a potential to influence alternative polyadenylation and termination of transcription [FAGHIHI AND WAHLESTEDT 2009]. NATs can also mask single-stranded RNA (ssRNA) and protect it from degradation by ribonucleases (RNases) (Fig. 1.3). *FGFR3-AS1*[16] forms dsRNA with fibroblast growth factor receptor 3 (*FGFR3*) mRNA. This dsRNA protects from RNase degradation and increases *FGFR3* mRNA stability and translation [J. SUN ET AL. 2016]. Overall, RNA masking is a sole NAT-RNA interaction that positively regulates mRNAs.

RNA interference

DsRNA, formed between an antisense and sense transcript, can be cleaved by an RNase III enzyme, Dicer [LAU ET AL. 2012]. Fragments of 21 to 23 nt are formed, [ELBASHIR ET AL. 2001; ZAMORE ET AL. 2000; H. ZHANG ET AL. 2002] known as an endogenous short interfering RNA (endo-siRNA) (Figs. 1 and 4 in OKAMURA AND LAI 2008]. One strand of endo-siRNA binds to Argonaute (AGO2) protein and forms RNA-induced silencing complex (RISC) [MACRAE ET AL. 2008; MATRANGA ET AL. 2005; MEISTER ET AL. 2004]. siRNA directs RISC to the sense mRNA, leading to mRNA cleavage and degradation (Fig. 1.3) [MARTINEZ ET AL. 2002; MATRANGA ET AL. 2005]. Dicer and AGO2, the key components of the RNA interference

[15] zinc finger E-box binding homeobox 2-antisense transcript 1
[16] fibroblast growth factor receptor 3-antisense transcript 1

(RNAi) machinery, are found in the nucleus and the cytoplasm [GAGNON ET AL. 2014; SCHRAIVOGEL ET AL. 2015; WEINMANN ET AL. 2009]. So, the NAT-induced RNAi is possible in both compartments [BEREZHNA ET AL. 2006; CLAYCOMB 2014; GAGNON ET AL. 2014; ROBB ET AL. 2005]. Some NAT-derived endo-siRNAs are produced in the *c-MYC* locus (*NAT6531*) [NAPOLI ET AL. 2017] and huntingtin (*HTT*) gene (*HTT-AS*) [CHUNG ET AL. 2011].

RNA editing

Adenosine to inosine (A-to-I) deamination is a change in nucleotide sequence catalyzed by ADAR (adenosine deaminase acting on RNA), an enzyme acting on nuclear dsRNA [BASS 2002]. Inosine pairs with cytosine and is recognized as guanosine, thus, this editing introduces changes of a primary sequence and subsequently codes for a nonfunctional protein. A-to-I editing is a NAT mechanism to induce nuclear retention of promiscuously edited mRNAs [Z. ZHANG AND CARMICHAEL 2001] or heterochromatin gene silencing (Fig. 1.3) [Q. WANG ET AL. 2005]. Prostate cancer antigen 3 (*PCA3*), a NAT, is the most specific biomarker in urine of human prostate cancer. *PCA3* promotes A-to-I editing and downregulates the expression of a tumor suppressor *PRUNE2* (prune homolog 2 with BCH domain), resulting in tumor growth [SALAMEH ET AL. 2015].

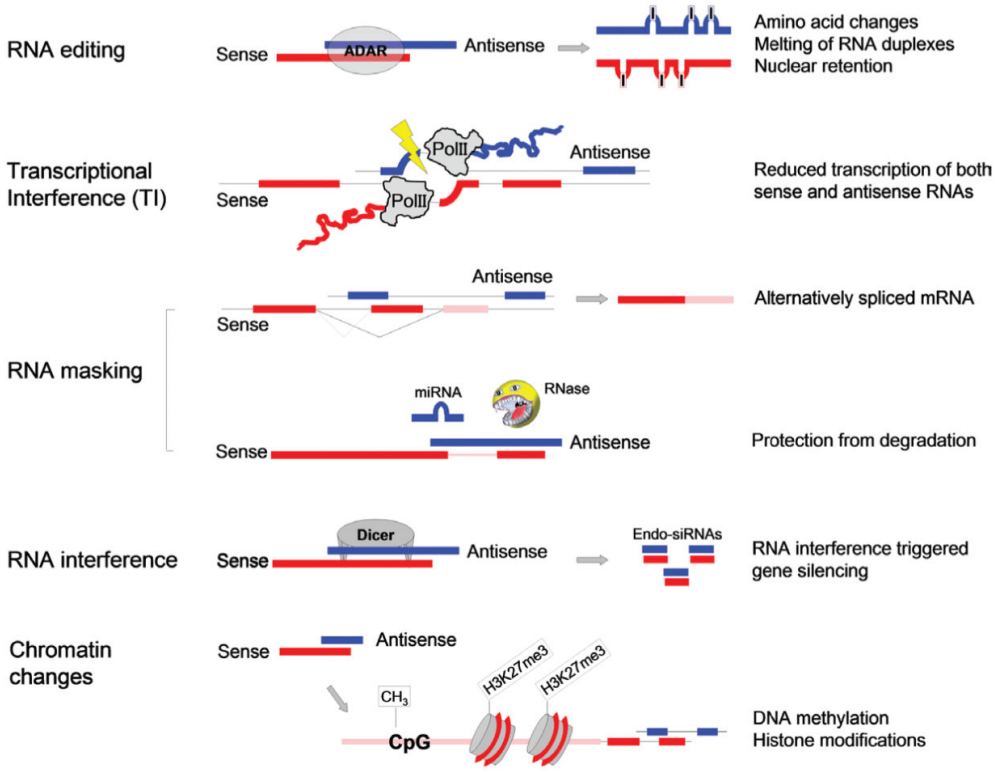


Figure 1.3: Mechanisms of action of antisense RNA transcripts in the nucleus. Mechanisms described in the text above. Image taken from WIGHT AND WERNER 2013. Copyright 2019, with permission from Portland Press Ltd.

1.5.1.5 NAT-RNA interactions in the cytoplasm

Masking miRNA binding sites

Similar to nuclear masking of splice and RNase-binding sites, NATs can bind to sense mRNAs in the cytoplasm and block the micro RNA (miRNA) binding site. In the Alzheimer's disease, NAT and miRNA compete for the binding site on *BACE1* mRNA. *BACE1-AS* binding blocks the binding of miR-485-5p. The inhibitory effect of miRNA on *BACE1* translation is blocked and *BACE1* mRNA is stabilized [FAGHIHI, M. ZHANG, ET AL. 2010; Fig. 1 in RASHID ET AL. 2016].

mRNA decay

mRNA degradation can be obtained by (STAU1[17])-mediated mRNA decay (SMD). STAU1, a dsRNA-binding protein, binds to the 3′ untranslated regions (UTRs) of translationally active mRNAs. STAU1 binding site (SBS) can be formed by imperfect base-pairing between an Alu element within the 3′ UTR of an SMD target and complementary Alu element within a specific lncRNA. Those specific lncRNAs, exclusively cytoplasmic, are called half STAU1-binding site RNAs (*½-sbsRNAs*). *½-sbsRNA1* base-pairs with the 3′ UTR of *SERPINE1*[18] mRNA. STAU1 stabilizes the duplex between the two halfs of SBS on *SERPINE1* mRNA and *½-sbsRNA1*. By recruiting STAU1 to trigger a *SERPINE1* mRNA decay, *½-sbsRNA1* contributes to the reduction of cell migration. Other *½-sbsRNA* members (2-4) induce an SMD in their targets in the same way [GONG AND MAQUAT 2011; Fig. 1 in RASHID ET AL. 2016].

1.5.1.6 Translational interference

NAT can interfere with the translation machinery and thereby prevent mRNA translation. The translation of transcription factor PU.1 is inhibited by its NAT. Antisense *PU.1* directly binds translation factors and stalls translation between the initiation and elongation step [EBRALIDZE ET AL. 2008]. Oppositely, SINEUPs are antisense RNAs that require the inverted SINEB2 sequence to UP-regulate translation [ZUCCHELLI ET AL. 2015]. Therefore, SINEUPs can increase synthesis of target proteins. In humans, *R12A-AS1* is an antisense to phosphatase 1 regulatory subunit 12A (*PPP1R12A*) and contains an effector domain FRAM[19], an Alu repeat. FRAM is important for binding of *PPP1R12A* mRNA and increasing its translation [SCHEIN ET AL. 2016]. *R12A-AS1* is similar to mouse *Uchl1* SINEUP [CARRIERI, CIMATTI, ET AL. 2012; CARRIERI, FORREST, ET AL. 2015].

[17] Staufen homolog 1
[18] serpin family E member 1, also known as plasminogen activator inhibitor type 1, PAI-1
[19] free right Alu monomer repeat element

1.5.1.7 NAT influencing protein functions

NATs can have direct regulatory functions on proteins. For example, NATs can modulate the activity of TFs and enzymes or influence the function of the signaling pathway components [reviewed by MARCHESE AND RAIMONDI 2017; RASHID ET AL. 2016]. Those NAT functions can be described by a decoy archetype.

Decoy archetype

NAT interacts with diverse regulators of gene expression and sequesters them away from binding to the target genomic loci, resulting in gene activation or silencing. NAT itself does not conduct additional functions except binding proteins (Fig. 1.4) [K. C. WANG AND CHANG 2011].

NF-κB[20] interacting long noncoding RNA (*NKILA*) influences the components of this signaling pathway. In the canonical pathway, the inhibitor of the NF-κB (IκB) is degraded when is phosphorylated. Then the NF-κB complex is activated and can translocate into the nucleus. *NKILA* forms hairpins and physically blocks the phosphorylation of IκB. Thus, the secondary structure of NAT suppresses the NF-κB activation, increases apoptosis and acts antimetastatic. *NKILA* is upregulated by NF-κB in a negative feedback loop [LIU ET AL. 2015].

NATs can likewise suppress TF activity. *PANDAR*[21] is a NAT transcribed from the CDKN1A[22] promoter in the antisense direction to *CDKN1A* mRNA. As a response to DNA damage, p53 binds to *CDKN1A* locus and activates *PANDAR* transcription. *PANDAR* binds transcription factor NF-YA[23] and pulls it away from its binding sites in proapoptotic genes [HUNG, Y. WANG, ET AL. 2011]. This way *PANDAR* attenuates apoptosis [HUNG, Y. WANG, ET AL. 2011; Z. LI ET AL. 2017; ZHAN ET AL. 2016].

Besides influencing protein functions, NATs can also regulate protein stability. DNA-damage-induced noncoding lncRNA (*DINO*) is another antisense transcript from the *CDKN1A* promoter region, induced by DNA damage-induced p53. As an RNA transcript, *DINO* directly binds to p53 and constitutes a positive feedback loop. *DINO* first stabilizes p53 and then guides p53 to its response elements on the downstream target genes, for example CDKN1A, and thus control cell cycle, DNA repair and apoptosis. Thus, *DINO* functions *in cis* and *in trans* [SCHMITT ET AL. 2016; Fig. 3b in MARCHESE AND RAIMONDI 2017].

Certain NATs act via more than one archetype. For example, *PANDAR* per-

[20] nuclear factor kappa-light-chain-enhancer of activated B cells
[21] p21 associated ncRNA DNA damage activated RNA
[22] cyclin dependent kinase inhibitor 1a
[23] nuclear transcription factor Y subunit alpha

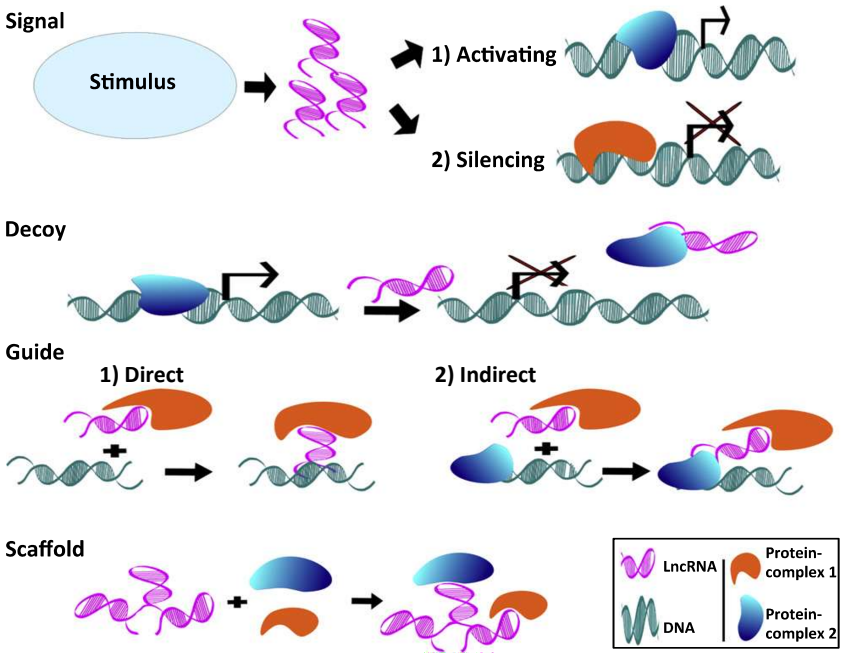


Figure 1.4: Four archetypes of lncRNA mechanisms. Signal NAT's transcription is a reaction to a certain stimulus or developmental stage and can consequently activate or silence gene transcription. In decoy archetype, NAT sequesters the regulatory proteins away from their binding site on DNA. In contrast, in guide archetype, NATs can guide regulatory proteins to their binding sites in a direct way or indirect, mediated by another protein. Scaffold archetype describes a NAT as a platform to which different regulatory proteins bind. NAT brings them to the certain DNA site. Added words "Direct" and "Indirect" to the original image. Image taken from B. HRDLICKOVA ET AL. 2014. Copyright 2019, with permission from Elsevier.

forms signal and decoy and *HOTTIP* signal and guide functions [K. C. WANG AND CHANG 2011]. *HOTAIR* serves as a scaffold and a guide for chromatin modifying complexes and an anatomic signal of posterior and distal sites [BALAS AND A. M. JOHNSON 2018; J. L. RINN, KERTESZ, ET AL. 2007; K. C. WANG AND CHANG 2011]. *MALAT1*[24] functions as a scaffold [reviewed by X. ZHANG, HAMBLIN, ET AL. 2017] and decoy [TRIPATHI ET AL. 2010]. On the other hand, different NATs can influence the same target [PENG ET AL. 2017].

1.6 Discovery of lncRNA *WISP1-AS1*

During the studies on the effects of ochratoxin A (OTA) on the primary culture of renal proximal tubule epithelial cells (RPTEC), Hennemeier et al. noticed an increased expression of the *WISP1*[25] RNA [2012]. All the efforts to confirm an upregulation of WISP1 at the protein level resulted in the absence of signal in Western blot (while antibodies were functional and positive control was giving

[24] metastasis associated lung adenocarcinoma transcript 1
[25] WNT1-inducible signaling pathway protein 1

a signal). This prompted Hennemeier and colleagues to investigate this RNA in more detail.

WNT1-inducible signaling pathway protein 1-antisense transcript 1 (*WISP1-AS1*) was not previously detected *in silico* and was not available in public databases. This might be because "standard RNA sequencing requires double-stranded cDNA synthesis, which erases RNA strand information, leading to expression quantification that is the sum of the expression of both transcripts of the cis-NAT pair" [WENRIC ET AL. 2017]. To detect antisense transcription, strand-specific RNA-sequencing would have to be employed [reviewed by R. HRDLICKOVA ET AL. 2017; LEVIN ET AL. 2010].

Since there was no information on the *WISP1-AS1* genomic location, primer walking and RACE[26] methods were used to obtain its full-length [HENNEMEIER 2014; HENNEMEIER ET AL. 2012]. The results have shown that *WISP1-AS1* is 2922 nt long and encoded from a part of intron 4, exon 5 and a part of 3′ UTR of *WISP1* gene (Fig. 1.5). By the ability to confirm *WISP1-AS1* length, and therefore the existence, a new lncRNA was discovered! It is an lncRNA because it is longer than 200 nt and not coding for WISP1 splice variant.

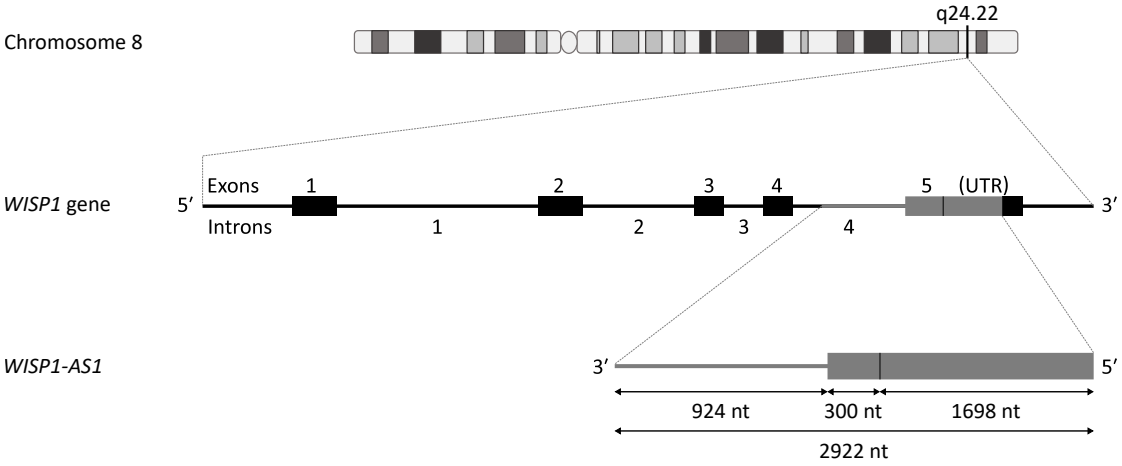


Figure 1.5: A schematic presentation of *WISP1* gene and *WISP1-AS1* genomic location and length. *WISP1* gene is located in humans on the chromosome 8, near the end of the longer arm (genomic location 8q24.22). *WISP1* gene consists of five exons. *WISP1-AS1* is transcribed from the part of intron 4 (924 nt), exon 5 (300 nt) and a part of the 3′ UTR (1698 nt). *WISP1-AS1* is 2922 nt long. See Table A.4 for *WISP1-AS1* sequence.

By separation of nuclear and cytoplasmic RNA, *WISP1-AS1* localization was detected predominantly in the nucleus [HENNEMEIER 2014; HENNEMEIER ET AL. 2012]. This further supported its noncoding nature due to compartmentalization away from the translation machinery [CABILI, DUNAGIN, ET AL. 2015; JI ET AL. 2015]. *WISP1-AS1* is an lncRNA with an increased expression after expo-

[26] rapid amplification of cDNA ends

sure to the mycotoxin OTA [Hennemeier 2014; Hennemeier et al. 2012]. Altered lncRNA expression after mycotoxin exposure *in human cells* was demonstrated by some studies. The very potent carcinogenic mycotoxin aflatoxin B1 upregulated lncRNA *H19* and increased the invasiveness of hepatocellular carcinoma [Lv et al. 2014].

1.7 Ochratoxin A (OTA)

Ochratoxin A (OTA) is a mycotoxin produced by fungi *Aspergillus ochraceus*, *Penicillium verrucosum* and other *Aspergillus* species. OTA-producing fungi are listed in Table 1 in Abrunhosa et al. 2010. OTA molecule contains a chlorinated dihydroisocoumarin moiety linked by a peptide bond to L-phenylalanine (Phe) (Fig. 1.6) [reviewed by El Khoury and Atoui 2010; Koszegi and Poór 2016].

In an aqueous solution, dependent on pH, OTA is present in three forms: nonionic, monoanionic (OTA^-) and dianionic (OTA^{2-}). Nonionic form is present in acidic solutions. With the pH increase, the molecule loses hydrogen (green in Fig. 1.6) on L-phenylalanine carboxylic group and becomes monoanionic. By losing hydrogen on the phenolic group, (green in Fig. 1.6) OTA becomes dianionic [Cagnasso et al. 2019; Poór et al. 2013 and Fig. 1 there within). Based on these chemical properties, OTA is very stable in acidic environments and overall a highly stable chemical [Trenk et al. 1971]. In crystalline structure, OTA has a melting point of 169 °C [Merwe, Steyn, and Fourie 1965; Merwe, Steyn, Fourie, et al. 1965], thus, it can endure high thermal processing [Trenk et al. 1971].

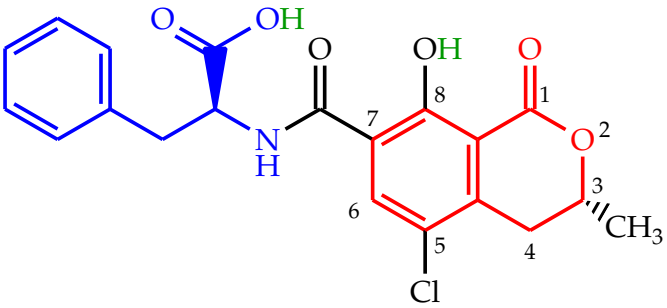


Figure 1.6: The molecule of ochratoxin A. Blue: L-phenylalanine residue; red: dihydroisocoumarin ring, green: acidic hydrogens.

1.7.1 Occurrence of natural OTA contamination

OTA contamination, detected in a wide variety of agricultural commodities (e.g. corn, barley, wheat, rice, coffee and cocoa beans, grapes) used for human or animal food, presents a significant problem worldwide [reviewed by Binder et al.

2007; Denli and Perez 2010; Duarte, Lino, et al. 2010; Malir et al. 2016]. Contamination occurs when fungi, species *Aspergillus* and *Penicillium*, produce OTA as a secondary metabolite, usually on the improperly stored grains in humid and warm places [reviewed by Cairns-Fuller et al. 2005; Duarte, Pena, et al. 2010; Magan and Aldred 2005]. Contamination of grains is concerning because OTA can be transferred through the food chain: from the animal feed to animals, and to humans by consuming animal products [reviewed by Abrunhosa et al. 2010; Denli and Perez 2010; Duarte, Lino, et al. 2012] or by direct consumption of contaminated cereals, fruits and processed products of plant origin [reviewed by Duarte, Lino, et al. 2012].

Once foodstuff is contaminated, it is very difficult to completely remove OTA [El Khoury and Atoui 2010; Koszegi and Poór 2016]. OTA is insensitive to heat treatments during cooking processes [Bullerman and Bianchini 2007; Kabak 2008], including baking bread [Scudamore et al. 2003; Vidal 2014]. Residual OTA contamination is observed after refinement processes like coffee roasting [Khaneghah et al. 2019; Stegen et al. 2001; reviewed by Leitão 2019] and very little after beer brewing [Beláková et al. 2011; reviewed by Anli and Alkis 2010]. Thus, avoiding OTA consumption by contaminated food is impossible! That's why, OTA can be detected in nm amounts in the plasma of healthy humans. Pfohl-Leszkowicz and Manderville 2007 in Table 1 reviewed OTA amounts in blood from healthy persons from 10 European countries between the 1970's and 1990's. European Food Safety Authority in Table 7 summarized OTA concentrations in human plasma between 1990's and 2000's [2006].

Autoclaving of contaminated food for 3 h [Trenk et al. 1971] or heating up to 250 °C [Boudra et al. 1995] did not completely destroy the OTA molecule. Only treatment in alkali conditions and high temperature can substantially destroy it [Trivedi et al. 1992]. Despite the methods to decompose the OTA molecule even up to 80–90 %, it should be kept in mind that toxic effect is (usually) not removed by degrading OTA; decomposed products might be just as toxic [Suárez-Quiroz et al. 2005].

1.7.2 OTA influence on renal cells

OTA is absorbed in the small intestines into the bloodstream [Kumagai and Aibara 1982] where it binds to different proteins [Schwerdt, Freudinger, Silbernagl, et al. 1999], with the highest affinity for the abundant albumin [Chu 1971; Perry et al. 2003]. Half-life of OTA in human serum is 35 days [Studer-Rohr et al. 2000]. OTA distributes to different organs: liver, skeletal muscles

and adipose tissue but kidney is the main target [reviewed by Ringot et al. 2006].

Kidneys contain the special transport mechanism, part of it being organic anion transporters (OATs), involved in the active cellular uptake of OTA. Anzai et al. reviewed transporters in the kidney responsible for OTA accumulation in the tubule cells [2010]. Damage by OTA is observable along the nephron[27]. The most affected proximal tubule cells [Gekle, Sauvant, et al. 2005; reviewed by Kuiper-Goodman and Scott 1989] absorb OTA from the blood on the basolateral side by OAT1 and OAT3 [X. Zhang, Groves, et al. 2004]. On the apical side OTA is reabsorbed from the tubule volume [Dahlmann et al. 1998; Zingerle et al. 1997; Fig. 1 in Anzai et al. 2010] by, for example, H^+-dipeptide cotransporter [Dahlmann et al. 1998; Schwerdt, Freudinger, Silbernagl, et al. 1998; Schwerdt, Gekle, et al. 1997] and OAT4 [Babu et al. 2002]. Thus, OTA accumulates in the proximal tuble cells, where it binds to cellular proteins [Schwerdt, Gekle, et al. 1997]. For a thorough review on OTA damage of renal structure and function, see Gekle, Sauvant, et al. 2005 and citations therein.

In accordance with OTA accumulation in the kidney, the main toxic effect of OTA is indeed nephropathy, observed in different animals [reviewed by Krogh 1992]. OTA is undoubtedly a cause of mycotoxic porcine nephropathy (MPN) [Elling and Moller 1973; Krogh et al. 1979] and (at least partially) a cause of human endemic nephropathies. The most studied is Balkan endemic nephropathy (BEN) [Abouzied et al. 2002; Ceović et al. 1985; Hult et al. 1982; Vrabcheva et al. 2004; Vukelic et al. 1992; reviewed by Fuchs and Peraica 2005; Pavlovic 2013; Stefanovic and Polenakovic 2009]. BEN appeared in certain parts of the Eastern Europe in areas with OTA-contaminated food (Fig. 1 in Stiborová et al. 2016] in farming households [Miletic-Medved et al. 2005]. The disease is characterized by the damage of the tubular epithelium, interstitial fibrosis, tubular and kidney atrophy [Vukelic et al. 1992]. The highest OTA concentration in the blood of BEN patient was 50 ng/ml [Radic et al. 1997]. Similar renal diseases observed in Tunisia [Grosso et al. 2003] and Egypt [Wafa et al. 1998] had maximum 8 and 10 ng/ml OTA in the blood of nephropathic patients.

One of the toxicological OTA effects [reviewed by Heussner and Bingle 2015; Kuiper-Goodman and Scott 1989; Pfohl-Leszkowicz and Manderville 2007 and citations there within] is carcinogenicity [reviewed by Pfohl-Leszkowicz and Manderville 2011]. OTA increases the incidence of renal adenoma and carcinoma in experimental animals [reviewed by Kuiper-Goodman and Scott 1989; Mally and Dekant 2009; Pfohl-Leszkowicz and Manderville 2011].

[27] nephron structure: Kriz et al. 1988

Higher incidence of urethro-renal cancers was also observed in BEN patients [CHERNOZEMSKY ET AL. 1977; FUCHS AND PERAICA 2005; MILETIC-MEDVED ET AL. 2005; STEFANOVIC, TONCHEVA, ET AL. 2006; VUKELIC ET AL. 1992].

Mechanisms of OTA action are described in different animals and human cell culture [reviewed by KOSZEGI AND POÓR 2016], although some are not well understood at the molecular level. At high OTA concentrations, Phe moiety of OTA inhibits Phe-tRNA synthetase activity and interferes with protein synthesis, decreasing total cellular protein [CREPPY ET AL. 1984]. Protein content was decreased after OTA treatment of mesangial [SCHWERDT, HOLZINGER, ET AL. 2009] and opposum [SAUVANT ET AL. 2005A] kidney cells in culture.

OTA activates the mitogen activated protein kinases (MAPK) [reviewed by CARGNELLO AND ROUX 2011] in the collecting duct and proximal tubule cells [GEKLE, SCHWERDT, ET AL. 2000; HENNEMEIER ET AL. 2012; SAUVANT ET AL. 2003, 2005A,B]. In particular, it activates ERK[28] and antagonistic JNK[29] and p38. ERK1/2 pathway plays specific roles in the control of cell proliferation and differentiation [reviewed by KESHET AND SEGER 2010] and mitosis [SHAPIRO ET AL. 1998]. JNK and p38 are involved in dedifferentiation, inflammation and cell death [MA ET AL. 2007; reviewed by GRYNBERG ET AL. 2017]. OTA promoted cell survival by activating ERK1/2. By activating JNK/p38, OTA induced apoptosis (and necrosis), markers of fibrosis and EMT; the changes observed in chronic interstitial nephropathies including BEN. Thus, JNK could be the underlying mechanism of OTA-induced nephropathies [GEKLE, SCHWERDT, ET AL. 2000; SAUVANT ET AL. 2003, 2005A,B].

In IHKE[30] cells, OTA influences cellular Ca^{2+} infux [BENESIC ET AL. 2000] and triggers: stimulated mitochondria, enhanced production of protons and ATP and decreased cytosolic pH (acidification). The latter two were also necessary for OTA-induced caspase-3 activation and apoptosis. No cytochrome c release was detected after OTA treatment, suggesting intact mitochondria [SCHWERDT, FREUDINGER, SCHUSTER, ET AL. 2004, 2003]. Acidification *can* naturally occur in the collecting duct of the kidney and form ideal conditions to enhance toxic OTA effects [SCHWERDT, FREUDINGER, SCHUSTER, ET AL. 2004]. OTA-induced, Ca^{2+}- dependent changes on mitochondrial membrane potential were: hyperpolarization in IHKE [EDER ET AL. 2000] and depolarization in MDCK-C7[31] cells [SCHWERDT, FREUDINGER, SCHUSTER, ET AL. 2004]. OTA-induced acidification was autonomous from aerobic glycolysis and lactate formation in IHKE cells [EDER ET AL. 2000;

[28] extracellular signal-regulated kinase
[29] c-jun N-terminal kinase, also known as stress-activated protein kinase (SAPK)
[30] immortalized human kidney epithelial
[31] Madin-Darby canine kidney clone 7

Schwerdt, Freudinger, Schuster, et al. 2003]. In summary, OTA toxicity is a result of cellular Ca^{2+}, functional mitochondria and acidification [Eder et al. 2000]. Other OTA mechanisms are reviewed by Koszegi and Poór 2016.

1.7.3 OTA influence on *WISP1-AS1* expression

OTA toxicity in renal cells seems to be mediated by *WISP1-AS1*. *WISP1-AS1* expression was significantly increased in RPTEC and HEK293T cells exposed to OTA in comparison with the respective controls. OTA-induced *WISP1-AS1* expression is specific for epithelial cells; it does not express in OTA-treated normal human lung fibroblasts (NHLF). Moreover, OTA-induced *WISP1-AS1* expression is time- and dose-dependent [Hennemeier et al. 2012]. Very important to remember is that OTA is the only known inducer of *WISP1-AS1*; the property we employ in the laboratory to induce endogenous *WISP1-AS1* expression.

Hennemeier et al. 2012 demonstrated that OTA must enter the cell to induce *WISP1-AS1* expression. Since OTA treatment influenced the activation of MEK/ERK signaling pathway, it was proposed that OTA probably binds to ERK in the cytoplasm (by unknown mechanism) which consequently induce *WISP1-AS1*. Other signaling pathways [JNK, p38, protein kinases A and C (PKA, PKC) and MSK1[32]] were not involved [Hennemeier et al. 2012].

OTA is known to induce carcinogenesis by various mechanisms [reviewed by Mally and Dekant 2009; Pfohl-Leszkowicz and Manderville 2011]. Additionally, lncRNAs are diferentially expressed and functional in tumors [reviewed by Gutschner and Diederichs 2012; Huarte 2015; Peng et al. 2017], including renal cell carcinoma (RCC) [Blondeau et al. 2015]. Histological types of RCC are: clear cell (80–90 %), papillary (10–15 %), chromophobe (4–5 %), and minor subtypes (< 1 %) [Ljungberg et al. 2019; Muglia and Prando 2015; Shuch et al. 2015]. OTA induces *WISP1-AS1* expression in renal epithelial cells. Clear cell renal cell carcinoma (ccRCC) 786-O cell line, derived from the epithelium of the renal proximal tubule was suitable to test potential *WISP1-AS1* involvement in carcinogenesis. 786-O cell line is reviewed by Brodaczewska et al. 2016.

1.8 PMA induction of *WISP1* mRNA expression

To understand experiments analyzing whether the transcripts from the *WISP1* locus influence each other's expression (Section 4.1.3.1), I hereby briefly introduce a chemical used in those experiments as well as the mode of *WISP1* mRNA

[32] mitogen and stress-activated kinase 1

transcription activation. Phorbol 12-myristate 13-acetate (PMA), also known as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) is a phorbol ester used in molecular biology as a PKC activator.

Activation of PKC family members can be triggered by natural extracellular signals (e.g. vasporessin [ALI ET AL. 1998], angiotensin II [NAGAHAMA ET AL. 2000; SHIBATA ET AL. 2014] and tumor necrosis factor (TNF) [SCHÜTZE ET AL. 1990], mediated by G protein-coupled or tyrosine kinase receptors [NEWTON 1997; NEWTON ET AL. 2016]. Conventional PKC activation involves phosphorylation and second messengers. Phosphorylation at certain amino acid residues produces stabilized and mature enzyme which localizes to the cytosol, but it is autoinhibited and catalitically inactive. G protein-mediated activation of phospholipase C (PLC) results in the generation of a second messenger diacylglycerol (DAG). Binding of DAG to the C1 domain and binding of phosphatidylserine (PS) and Ca^{2+} to the C2 domain of PKC promote the pseudosubstrate removal from the active site. Activated PKC is ready to catalize phosphorylation of various regulatory proteins and boost downstream signal transduction [reviewed by CALLENDER AND NEWTON 2017; NEWTON 1997; STEINBERG 2008].

The phorbol esters, including PMA, mimic the action of DAG. PMA binds to C1 domain of PKC with higher affinity than DAG [MOSIOR AND NEWTON 1996] and induces PKC binding to PS in the membrane but in Ca^{2+}- and DAG-independent manner [MOSIOR AND NEWTON 1996; VANDENBARK ET AL. 1984; reviewed by NEWTON 1997]. PMA-activated PKC can phosphorylate and activate a TF CREB[33] [GUO ET AL. 2012]. Different databases[34], available at https://amp.pharm.mssm.edu/Harmonizome/gene/WISP1 [ROUILLARD ET AL. 2016] predict CREB to have binding sites in the *WISP1* promoter. Indeed, CREB was shown to be important in the activation of *WISP1* transcription [XU ET AL. 2000]. WISP1 belongs to the CCN gene family [MALIK ET AL. 2015]. WISP1 was renamed to CCN4 (cellular communication network factor 4) to obtain more uniform nomenclature [PERBAL ET AL. 2018]. WISP1 plays important roles in skeletal development and osteogenesis [FRENCH ET AL. 2004; ONO ET AL. 2011], wound healing and tissue repair [FRENCH ET AL. 2004; QUIROS ET AL. 2017], as well as in pathological conditions like cancer [reviewed by DHAR AND RAY 2010; GURBUZ AND CHIQUET-EHRISMANN 2015].

To summarize, PMA-induced PKC phosphorylates CREB which binds to the *WISP1* promoter and activates mRNA transcription. Therefore, PMA was used as a positive control to experimentally induce *WISP1* mRNA.

[33] cAMP-response element binding protein
[34] Databases: JASPAR Predicted Transcription Factor Targets; MotifMap Predicted Transcription Factor Targets; TRANSFAC Curated Transcription Factor Targets

2 The aims of the study

To fill the gap in knowledge about OTA influence on renal epithelial cells, it is important to investigate *WISP1-AS1* functions and find an exact mechanism of OTA action on cells via *WISP1-AS1*. Therefore, this study was initiated because the characteristics and function(s) of *WISP1-AS1* were not investigated in great detail, and many questions remained to be answered.

Prior to this study, only some *WISP1-AS1* molecular characteristics were known, for example, nuclear localization, length and non-protein coding feature. The aim of this study was to further characterize *WISP1-AS1* at molecular level. Main tasks were to identify *WISP1-AS1* orientation in which it transcribes, relative to *WISP1* mRNA. Second, it was of interest to confirm *WISP1-AS1* expression, length and orientation by Northern blot. *In silico* methods were employed to find an evidence of *WISP1-AS1* expression and a support for its non-coding function.

When *WISP1-AS1* was discovered to be transcribed in antisense, next important aims were to analyze *WISP1-AS1* transcriptional landscape. The task was to investigate the relation between *WISP1-AS1* and *WISP1* mRNA because it is possible that two convergent overlapping transcripts influence each other's expression. Since *WISP1-AS1* transcribes in antisense from the end of *WISP1* gene, it was important to analyze whether *WISP1-AS1* might be a transcriptional extension from an upstream gene, *NDRG1*.

HEK293T cells treated with OTA are known to induce *WISP1-AS1* expression. On the other hand, OTA is suspected to contribute to the incidence of urothelial and renal cancers in humans. Therefore, the aim was to analyze *WISP1-AS1* expression in renal cancer cell line (treated and untreated with OTA) to investigate its expression in cancer cells and potential involvement in carcinogenesis.

To study the function of *WISP1-AS1*, the aim was to establish a knockdown tool to modulate *WISP1-AS1* expression. In the conditions of reduced *WISP1-AS1* expression, the aim was to investigate the influence of *WISP1-AS1* on cell transcriptome and validate results by PCR methods. Through the analysis of results, the aim was to find gene ontology or a specific pathway/gene which would be investigated in more detail and which could help in revealing the molecular function of *WISP1-AS1*. Finally, the aims were to accomplish *WISP1-AS1* influence on cellular energy metabolism (glucose consumption and lactate production) and cell death (apoptosis and necrosis).

3 Materials and Methods

3.1 Chemicals

Dulbecco's minimal essential medium (DMEM)/Ham's F-12 medium (1:1 mixture; FG 4815), Roswell Park Memorial Institute (RPMI) 1640 medium (FG 1215) and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). Water (molecular biology reagent), OTA and PMA were provided by Sigma-Aldrich (Darmstadt, Germany). Antisense oligonucleotides (ASOs) were obtained from Exiqon (Vedbeak, Denmark). Other purchased materials are specified in the text.

3.2 Basic molecular biology methods

3.2.1 Cell culture

Human embryonic kidney 293T (HEK293T; ATCC® CRL3216™) cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HEK293T cells were grown in DMEM/Ham's F-12 medium supplemented with 10 % FCS at 37 °C and 5 % CO_2. When the confluency of 80–90 % was reached, medium was changed to serum-free DMEM/Ham's F-12 and the cells were cultivated for 24 h. Subsequently, cells were treated with 100 nM OTA or 1 µM PMA in final concentrations or with OTA + PMA in serum-free media. Incubations were carried out without media change for 24 h or 48 h, described in individual experiments. Control cells were grown in serum-free medium without OTA.

Clear cell renal cell carcinoma (ccRCC) cell line 786-O (ATCC® CRL-1932™) was purchased from ATCC (Manassas, VA, USA). 786-O cells were grown in RPMI 1640 medium supplemented with 10 % FCS until confluency, then for 24 h in medium without FCS. Next, half of the Petri dishes were treated with 100 nM OTA in serum-free medium for 48 h and another half were grown as controls in serum-free medium without OTA.

Further cell culture experiments are described in Sections 3.2.7 and 3.3.2.

3.2.2 RNA isolation and quality control

InviTrap® Spin Tissue RNA Mini Kit (Stratec, Berlin, Germany) was employed for total RNA isolation by following manufacturer's instructions. Polyadeny-

lated RNA fraction was isolated by using Oligotex Kit (Qiagen, Hilden, Germany).

To isolate RNA for RNA sequencing (RNA-seq), TRIzol® Reagent (Invitrogen, Life Technologies, Darmstadt, Germany) and chloroform (Sigma-Aldrich, Darmstadt, Germany) were used according to TRIzol Reagent protocol[1]. Subsequently, RNA was precipitated in 2 volumes of 100 % ice-cold ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) at −20 °C overnight. The next day, tubes were centrifuged at 12 000× g and 4 °C for 30 min. After removing the supernatant, the pellets were resuspended in 75 % ice-cold ethanol and centrifuged at 12 000× g and 4 °C for 10 min. The dried RNA pellets were resuspended in 30 µl water.

In the subsequent treatment, DNA contamination was reduced by deoxyribonuclease I [DNase I; New England Biolabs (NEB), Frankfurt am Main, Germany]. Therefore, to 30 µl of RNA was added 3.6 µl of 10× DNase I reaction buffer (NEB, Frankfurt am Main, Germany) and 2.4 µl of DNase I (2 U/µl). Tubes were incubated in a thermocycler TProfessional (Biometra, Göttingen, Germany) at 37 °C for 30 min followed by 75 °C for 10 min to inactivate the enzyme. Afterwards, tubes were centrifuged at 11 000× g and 4 °C for 2 min and the supernatant was transferred into a new tube. Precipitation step was repeated as described above, but with the incubation at −20 °C for 2 h.

RNA concentrations were measured by spectrophotometer NanoVue™ Plus (GE Healthcare, Berlin, Germany). High RNA purity was assessed from both A260/A280 and A260/A230 ratios around 2.

For methods where RNA integrity was of an exceptional importance (e.g. Northern blot, RNA-seq), and when RNA was frozen longer than 12 months, 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) was used to measure RNA concentration and quality. All RNA was of a high quality obtained from the RNA integrity number (RIN) on average 9 out of 10.

All RNA samples were stored at −80 °C.

3.2.3 cDNA preparation

To remove genomic DNA contamination, total RNA was thoroughly treated by DNase I (NEB, Frankfurt am Main, Germany). This step was crucial because any traces of genomic DNA can produce false positive results in a PCR. Two types of reactions were prepared on ice by mixing either 1 µg of total RNA or 100 ng of polyadenylated RNA with water up to the 11.2 µl, 1.4 µl of 10x DNase I reac-

[1] https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf

tion buffer (NEB, Frankfurt am Main, Germany) and 1.4 µl of DNase I(2 U/µl). Tubes were incubated in a thermocycler TProfessional (Biometra, Göttingen, Germany) at 25 °C for 30 min, followed by 37 °C for 30 min and with final 75 °C for 10 min to inactivate the enzyme.

After DNase I treatment, each sample was separated into two tubes: 10 µl for reverse transcription with reverse transcriptase (RTase) and 4 µl for control reaction without RTase. The latter served to detect DNA contamination remained after DNase I treatment. Reverse transcription reactions of RNA into first-strand cDNA were prepared with a SuperScript™ II Kit (Invitrogen, Life Technologies, Darmstadt, Germany), as shown in Table 3.1. Tubes were incubated in a thermocycler TProfessional (Biometra, Göttingen, Germany) at 25 °C for 5 min, followed by 46 °C for 30 min and final 95 °C for 5 min to inactivate RTase.

Table 3.1: Reverse transcription with reverse transcriptase and control reaction without reverse transcriptase

V(1× RTase), µl	Reagent	V(1× no-RTase), µl
14.80	Water	6.09
7.14	5× First-strand buffer (Invitrogen)	2.85
0.36	100 mM DTT (Invitrogen)	0.14
1.41	10 mM-each-dNTP mix (PeqLab)	0.56
1.00	10 mM primer (see list below)	0.38
0.71	40 U/µl RNase inhibitor (RNase Out™, Invitrogen)	0.28
0.36	200 U/µl RTase SuperScript™ II (Invitrogen)	–
10.00	DNase I-treated RNA	4.00
35.78	Total volume	14.30

Reverse transcription was performed with different primers:

1. Random hexamers (3 µg/µl, Invitrogen) for standard cDNA preparation,
2. 18-mer oligonucleotide deoxythymine (oligo$(dT)_{18}$) primer to reverse transcribe only polyadenylated RNA,
3. Strand-specific primer to obtain *WISP1-AS1* orientation:
   (a) sense-orientation-specific primer in *WISP1* 3′ UTR (5′ `CACACCCCTACCTGAACCTG` 3′)
   (b) antisense-orientation-specific primer in *WISP1* intron 4 (5′ `GTGTCCCCTGAGTGTGGAGT` 3′)
4. No primer to analyze primer-dependent RTase function.

3.2.4 Real-time quantitative polymerase chain reaction (qPCR)

Real-Time qPCR reactions were performed on a CFX96 Touch Real-time PCR Detection System (Bio-Rad, Munich, Germany) and 7900HT Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Karlsruhe, Germany). Reactions performed on the CFX96 system contained 10 µl 2× Platinum® SYBR® Green qPCR SuperMix without UDG (Invitrogen, Life Technologies, Darmstadt, Germany), 1 µl of 10 mM forward and 1 µl of 10 mM reverse primer, 1 µl of 750 ng cDNA and water up to the final volume of 20 µl. For reactions performed on the 7900HT system, all volumes except cDNA were reduced by 40 % to get a total volume of 12 µl. qPCR primers were designed in a *Primer3Plus*[2] and *Primer-BLAST*[3] and purchased from Eurofins Scientific (Ebersberg, Germany).

On both qPCR systems, amplification was performed with initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 60 °C[4] for 30 s and extension at 72 °C for 20 s. From the results ΔΔCt was calculated as $\Delta Ct_{control}$ - $\Delta Ct_{treated}$. Fold change in gene expression in a treated sample relative to a control was expressed as $2^{\Delta\Delta Ct}$ normalized to a reference gene (either *18S* rRNA, *ACTB* or *GAPDH*).

qPCR products were mixed with a 10× loading dye CoralLoad PCR buffer (Qiagen, Hilden, Germany) and together with DNA ladder mix (100 to 10 000 bp, PeqGOLD, VWR, Dresden, Germany) analyzed by 1–2 % agarose gel electrophoresis. Bands were stained by ethidium bromide and visualized in an UV transilluminator and BioDoc-It™ Imaging System (UVP via VWR, Dresden, Germany). Where it was necessary to determine amplified sequence, bands were excised from the gel following the manual of QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sent for DNA sequencing (Eurofins Scientific, Ebersberg, Germany).

3.2.5 TaqMan PCR

TaqMan assays were used with primers and fluorescently labeled probes. On the 5′ end, *WISP1-AS1*-specific probe was labeled with a reporter, 6-fluorescein amidite (FAM™) and *WISP1* mRNA-specific probe with hexachlorofluorescein (HEX™). Both probes were labeled with a Black Hole Quencher 1 (BHQ®-1) at the 3′ end (see Table A.3 for sequences).

Reactions were prepared according to Table 3.2 and performed on a CFX96

[2] described by Untergasser et al. 2007
[3] described by Ye et al. 2012
[4] unless stated different in Table A.3

PCR machine (Bio-Rad, Munich, Germany) under the following cycling conditions: initial 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 59 °C for 60 s.

Table 3.2: TaqMan PCR reaction

Reagent	V(1× reaction), µl
2× iTaq™ Universal Probes Supermix (Bio-Rad)	6.0
Water	3.2
750 ng first-strand cDNA	1.0
10 mM Forward *WISP1-AS1* (intron 4) primer	0.3
10 mM Reverse *WISP1-AS1* (intron 4) primer	0.3
10 mM *WISP1-AS1* FAM-labeled probe	0.3
10 mM Forward *WISP1* (exon 1 - exon 2) primer	0.3
10 mM Reverse *WISP1* (exon 1 - exon 2) primer	0.3
10 mM *WISP1* HEX-labeled TaqMan probe	0.3
Total volume	12.0

3.2.6 Droplet digital™ PCR

For absolute quantification of RNA molecules, QX200™ Droplet Digital™ PCR (ddPCR) System (Bio-Rad, Munich, Germany) was used, following the applications guide.

In short, reaction mix was prepared with a ddPCR Supermix for EvaGreen (i.e. for PCR primer pairs) or a ddPCR Supermix for Probes (i.e. for TaqMan probes), as shown in Table 3.3. During droplet generation, 20 µl of a reaction mix was combined with 70 µl of QX200 Droplet Generation Oil for EvaGreen or for Probes. Droplets were generated in a Droplet Generator machine and transferred into a 96-well plate.

PCR amplification was performed in a C1000 Touch™ Termal Cycler (Bio-Rad, Munich, Germany) at the following conditions: initial 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 59 °C for 60 s. For the reactions with EvaGreen supermix, enzyme deactivation was performed at 98 °C for 10 min.

After PCR amplification, droplets were counted by Droplet reader and QuantaSoft™ Software. The amount of transcript per µg of total RNA was calculated from the results.

Table 3.3: Preparation of ddPCR reactions

(**a**) EvaGreen

Reagent	V(1×), µl
2× ddPCR Supermix for EvaGreen (Bio-Rad)	11.00
10 mM forward primer	0.22
10 mM reverse primer	0.22
Water	8.36
350 ng cDNA	2.20
Total volume	22.00

(**b**) Probes

Reagent	V(1×), µl
2× ddPCR Supermix for Probes (Bio-Rad)	11.00
10 mM *WISP1-AS1* (intron 4) forward primer	0.55
10 mM *WISP1-AS1* (intron 4) reverse primer	0.55
10 mM *WISP1-AS1* FAM-labeled TaqMan probe	0.55
10 mM *WISP1* (exon 1 - exon 2) forward primer	0.55
10 mM *WISP1* (exon 1 - exon 2) reverse primer	0.55
10 mM *WISP1* HEX-labeled TaqMan probe	0.55
Water	4.50
350 ng cDNA	3.20
Total volume	22.00

3.2.7 *WISP1-AS1* knockdown by LNA™ GapmeR antisense oligonucleotides (ASOs)

Locked nucleic acid (LNA)™ GapmeR antisense oligonucleotides (ASOs) were selected to knockdown *WISP1-AS1*. LNA™ GampeR ASO has modified RNA-like bases (i.e LNA) on each end and DNA bases in the middle (i.e. the "gap"). Furthermore, the whole molecule of ASO comprises of a chemically modified phosphorothioate backbone to protect against degradation by nucleases. ASOs bind to the RNA target in the nucleus. Consequently, DNA part of the molecule induces RNase H-dependent degradation of the target while ASO "survives" the process [reviewed by Chan et al. 2006].

Five LNA™ GapmeR ASOs of 16 nt and complementary (i.e. antisense) to intron 4 of *WISP1-AS1* were designed by and purchased from Exiqon (Vedbeak, Denmark). Confluent HEK293T cells were transfected in a 24-well plate. Antisense ASOs (i.e. *WISP1-AS1* ASOs) were added in a final total amount of 150 nM/well to 12 wells, and 150 nM/well of a scramble ASO to remaining 12 wells. To facilitate ASO uptake by the cells, Lipofectamine™ 2000 (Invitrogen, Life Technologies, Darmstadt, Germany) was used in a final concentration of 2 µg/well. Cells were incubated 5 h to allow ASOs to enter the nucleus, then half of each ASO group was treated with 100 nM OTA in final concentration. Groups are described in Table 3.4. Cells were incubated for 48 h without media change. Subsequently, *WISP1-AS1* knockdown was evaluated in total RNA by qPCR and ddPCR.

ASOs directed against sense-oriented *WISP1-AS1* (i.e. sense ASOs) were used to show that only antisense ASOs knockdown *WISP1-AS1*. The cell culture procedure was identical to antisense ASOs. See Table A.1 for ASO sequences.

Table 3.4: Treatment groups for *WISP1-AS1* knockdown by ASOs

Group	Anticipated outcome on *WISP1-AS1* expression
OTA/scramble ASO	*WISP1-AS1* expression is induced by OTA
OTA/*WISP1-AS1* ASOs	*WISP1-AS1* expression is induced by OTA but knockdown by ASOs reduces *WISP1-AS1* expression
Control/scramble ASO	Low basal *WISP1-AS1* expression
Control/*WISP1-AS1* ASOs	Low basal *WISP1-AS1* expression. ASOs do not have many targets to knock down

3.2.8 Northern blot

364 nt of *WISP1* intron 4 [primer pair *WISP1* intron 4 (V); see Table A.3 for sequences] was amplified by Platinum™ *Pfx* DNA Polymerase (Thermo Fisher scientific, Karlsruhe, Germany) according to manifacturer's protocol to produce a blunt-end PCR product. This product was then ligated in sense and in antisense orientation into a modified pUC18 plasmid (a generous donation by Dr. Selma Gago Zachert[5]), digested at EcoRV site. Ligation was performed according to Table 3.5 in insert-to-vector molar ratio 5:1. Reactions were incubated in a thermocycler TProfessional (Biometra, Göttingen, Germany) at 22 °C for 60 min.

Table 3.5: Ligation reaction

Reagent	V(1× reaction), µl
15 ng insert in water	10.2
10× T4 DNA ligase buffer (NEB)	1.5
50% w/v Polyethylene glcyol (PEG) 4000 solution (Sigma-Aldrich)	1.5
20 ng EcoRV-digested modified vector pUC18	1.0
5 U/µl T4 ligase (NEB)	0.8
Total volume	15.0

Following, 5 µl of the ligation reaction was transformed into 50 µl One Shot™ TOP10 Chemically Competent *E. coli* cells (Invitrogen, Life Technologies, Darmstadt, Germany) according to their protocol. Isolation of plasmid DNA was performed by Invisorb® Spin Plasmid Mini Two (Stratec, Berlin, Germany). Diagnostic digest of isolated plasmids was performed with two restriction enzymes according to the reaction conditions by NEB (Frankfurt am Main, Germany); NheI that cuts only once in an insert and HindIII that cuts only once in a vector. By analysis of different patterns of bands on the agarose gel, it was distinguished which plasmids carry inserts and in which orientation.

10 µg of plasmids carrying inserts were linearized with XbaI (NEB, Frankfurt am Main, Germany) restriction enzyme and then purified by Roti®-

[5] Leibnitz Institute of plant biochemistry, Halle (Saale), Germany

Phenol/Chloroform/Isoamyl alcohol, pH 7.5–8.0 (Roth, Karlsruhe, Germany). DNA concentration was determined with a spectrophotometer NanoVue™ Plus (GE Healthcare, Berlin, Germany). Both sense and antisense probes were *in vitro* transcribed twice:

1. in the presence of biotin-16-UTP (Roche Diagnostics, Mannheim, Germany) to generate biotinylated probes
2. without biotinylated UTP to produce riboprobes that will serve as hybridization controls.

Reactions were set as shown in Table 3.6, carried out at 37 °C for 2.5 h.

Table 3.6: *In vitro* transcription reactions with and without biotin-labeled UTP

Reagent	V(1× reaction), µl
1 µg purified linearized plasmid in water	5.7
5× Transcription buffer	2.8
40 U/µl RNase inhibitor (Rnase Out, Invitrogen)	0.5
20 U/µl T7 RNA polymerase (Thermo scientific)	2.0
10 mM each ATP, CTP, GTP, UTP (PeqLab) *or* 10 mM each ATP, CTP, GTP, 6.5 mM UTP, 3.5 mM biotin-16-UTP	3.0
Total volume	14.0

Following, DNA template was degraded by incubation with 2 µl of DNase I (2 U/µl, NEB, Frankfurt am Main, Germany). Riboprobes were precipitated by 2.5 M lithium chloride in final concentration at −20 °C overnight. The purity and size of biotinylated probes were confirmed by 5 % polyacrylamide gel electrophoresis. Slight retention in mobility of biotinylated probes was observed in comparison with non-biotinylated *in vitro* transcribed probes.

Total RNA, isolated from OTA-treated and untreated ccRCC cells, was loaded on 0.7 % agarose-1 M urea gel. Separation by electrophoresis was carried out at 80 mA for 2 h in 1× TBE buffer. During gel preparation, 5 µl of G Stain (Serva Electrophoresis, Heidelberg, Germany) was added into the gel to visualize RNA separation during electrophoresis. RiboRuler High range RNA ladder (Thermo Fisher Scientific, Karlsruhe, Germany) was used for size determination. RNA was blotted on the nylon membrane by capillary transfer for 20 h, followed by UV-crosslinking of RNA to the membrane with 1.2 J/cm^2 for 2 min.

After 1 h pre-hybridization, the membrane was hybridized with 800 ng of biotinylated sense or antisense probe (see Table A.2 for sequences) for 22 h. Both pre-hybridization and hybridization were carried out at 68 °C in the ULTRAhyb®

Ultrasensitive Hybridization Buffer (Thermo Fisher Scientific, Karlsruhe, Germany) with constant rotating. Detection was performed according to Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Fisher Scientific, Karlsruhe, Germany). Chemiluminescent signal was recorded by digital imaging system (ImageQuant LAS 4000, GE Healthcare, Freiburg, Germany), using ImageQuant program and increment method with up to 30 min interval time.

Double-distilled water was treated with 0.1 % v/v diethyl pyrocarbonate (DEPC; Sigma-Aldrich, Darmstadt, Germany) and used for preparing Northern blot buffers. Membranes hybridized with *WISP1-AS1* probes were stripped with 0.5 % SDS buffer [2.5 g SDS pellets (Roth, Karlsruhe, Germany) dissolved in 500 ml DEPC-treated water] at 60 °C with constant shaking during 60 min. Pre-hybridization and hybridization with 500 ng biotinylated *ACTB* mRNA probe (Exiqon, Vedbeak, Denmark; see Table A.2 for sequences) were carried out as described above.

To remove RNases, laboratory dishes were soaked in 5 % SDS buffer (50 g SDS pellets dissolved in 1 L double-distilled water) and rinsed with DEPC-treated water. Work surface was cleaned with RNaseZAP™ (Applied Biosystems, Thermo Fisher Scientific, Karlsruhe, Germany).

3.3 RNA-sequencing data

3.3.1 Initial data processing

For RNA-seq, four groups of samples were produced:

1. OTA/scramble ASO,
2. OTA/*WISP1-AS1* ASOs,
3. control/scramble ASO,
4. control/*WISP1-AS1* ASOs.

Groups are described in Table 3.4.

At the Core Unit DNA Technologies [6], libraries were prepared by fragmenting RNA, synthesizing cDNA and adding a sequencing adapter on each end; cDNA was attached onto the 'flow cell' through adapters. Clusters were generated by bridge-amplification of cDNA, followed by sequencing-by-synthesis on a HiSeq2000 sequencer (Illumina). The total raw RNA sequencing reads were filtered by base call quality scores (*FastQC*). Adapter sequences were clipped (*cutadapt*) and the remaining reads were aligned (or mapped) to the Ensembl

[6] Medical Faculty, University Leipzig, Liebigstraße 21, 04103 Leipzig, Germany

human reference genome release 87 (*Bowtie, TopHat, SAMtools*). Quality check showed successful alignment, with 76–82 % aligned sequences. Aligned reads were visualized in Integrative Genomics Viewer (IGV)[7]. Reads per gene were quantified and annotated (*R*). Normalization (reads within genes calculated relative to the total number of reads and entire RNA repertoire) was performed to remove variation between samples (*edgeR*).

Differential expression (DE) of genes was calculated in each group against the control/scramble ASO group (*DEseq2*). Statistical significance named false discovery rate (FDR) was calculated and corrected for multiple testing (*DEseq2*). For further calculation, genes with FDR below 0.005 (expecting five in 1000 genes to be false positive) were used.

Protein coding transcripts were sorted by DE: only genes in the OTA/scramble ASO group compared to the control/scramble ASO group were allowed to show DE (1 = upregulated; -1 = downregulated). At the same time, genes should show no change (DE = 0) in control/*WISP1-AS1* ASOs nor OTA/*WISP1-AS1* ASOs group. Following this, upregulated and downregulated genes were sorted by two parameters:

1. The abundance of a transcript in RNA sequencing, expressed as fragments per million (FPM)
2. Fold change (FC), according to the differential expression.

Genes with FC > |1.5| and FPM > 10 were further analyzed for the enrichment:

1. in gene ontology (GO) categories 'biological process', 'cellular component' and 'molecular function' by *g:Profiler*[8] and *GOrilla*[9].
2. of transcription factor binding sites (TFBSs) in promoter regions by *pscan*[10] and *g:Profiler*. In *pscan*, the region from -950 to +50 bp from the transcription start site (TSS)[11] was analyzed and the JASPAR 2016 database [Mathelier et al. 2016] was used. In *g:Profiler*, TFBSs in gene promoters were computationally predicted, based on the TRANSFAC database.

3.3.2 Promoter activity assays

The activities of TFs EGR-1 and E2F, whose TFBSs were *in silico* predicted to be enriched in DE genes were tested by Cignal™ reporter assays (Qiagen, Hilden,

[7] described by Robinson et al. 2011
[8] described by Reimand et al. 2016
[9] described by Eden et al. 2009
[10] described by Zambelli et al. 2009
[11] TSS is labeled as a base pair zero.

Germany). Reporter assays contained expression vectors with cloned repeats of response elements for TFs (i.e. TFBSs) and a reporter gene.

EGR-1 reporter assay contained TFBS sequence (CGCCCCCGCG) and a monster green fluorescent protein (MGFP) reporter protein. Transfection control contained constitutively expressing red fluorescent protein (RFP). To measure E2F activity, the Dual-Luciferase® Reporter (DLR™) assay was employed, comprised of E2F response element (TTTCGCGGGAAA) with Firefly luciferase reporter and constitutively expressing Renilla luciferase as an internal control. To analyze the activity of TF AP-1 whose TFBS was not enriched in DE genes, AP-1 reporter assay with response element (TGAGTCAG) and MGFP reporter was used.

To accomplish OTA influence on the activity of TFs, HEK293T cells were transfected with 1 µg of EGR-1, E2F or AP-1 vector and incubated with different concentrations of OTA (0, 1, 10, 100 and 1000 nM) for 24 h.

To analyze *WISP1-AS1* involvement in TF activity changed by OTA, confluent HEK293T cells were transfected in 60 mm Petri dishes with 1 µg of either:

1. E2F vector (two Petri dishes, for 24 h and 48 h luciferase measurements) or
2. EGR-1 vector and 0.1 µg of RFP vector (one Petri dish, for Cytation™ 3 Cell Imaging).

Transfection was facilitated with 26 µg/ml Polyfect Reagent (Qiagen, Hilden, Germany). After 24 h, cells were trypsinized and seeded in three 96-well plates (48 wells/plate). Each plate contained untransfected cells as a negative control (6 wells) and transfected cells incubated in a DMEM/Ham's F-12 medium supplemented with 10 % FCS as a positive control (6 wells). Outer wells were left empty.

After 24 h, 24 wells on each plate were treated with 150 nM ASOs for *WISP1-AS1* knockdown and 24 wells with scramble ASO. From each ASO group, 12 wells were treated with 100 nM OTA and 12 wells were left untreated.

EGR-1 activity was measured by Cytation™ 3 Cell Imaging Multi-Mode Reader and automated image analysis (Gen5 software, BioTek, Bad Friedrichshall, Germany) 6, 24 and 48 h after OTA addition. E2F was measured by the Dual-Luciferase® Reporter (DLR™) assay system (Promega, Manneim, Germany) and luminescence was read on a microplate reader (Infinite 200, Tecan, Crailsheim, Germany) after 24 and 48 h.

3.3.3 Gene validation

Some genes that were differentially expressed in RNA-seq were validated by qPCR and ddPCR. 28 genes were chosen with FC > |1.5| and FPM > 20 in the OTA/scramble ASO group or the control/scramble ASO group. See Table A.3 for primer sequences.

3.4 Cell death

3.4.1 Cell culture treatment and cell lysis

HEK293T cells were treated with OTA and ASOs as described in Section 3.2.7. After 48 h incubation, cell culture medium was collected into 1.5 ml tubes for a measurement of lactate dehydrogenase (LDH) release. Cells were washed with ice-cold 1× phosphate-buffered saline (PBS) and lysed on ice in 100 µl/well of lysis buffer (20 mM MOPS, 0.01 % Triton-X-100, 10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5). Cells were scraped with a 1 ml-pipette tip and collected into 1.5 ml tubes. Tubes were centrifuged at 16 000× g and 4 °C for 10 min. Supernatant (i.e. cell lysate) was transferred into a new 1.5 ml tube and used to determine protein concentration, caspase activity and intracellular LDH activity.

3.4.2 Determination of protein content

To quantify total cellular proteins, Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Karlsruhe, Germany) was used following the 'Microplate Procedure'. The method is based on a Biuret test which detects protein reduction of cupric ion ($Cu^{2+} \longrightarrow Cu^{+}$). The kit's reagent contains bicinchoninic acid (BCA) that can chelate Cu^{+}; the produced complex is proportional to the protein concentration.

Modification of the protocol included different volumes of bovine serum albumine (BSA) standards (0, 100, 300, 500 and 700 µg/ml, 15 µl in duplicates), cell lysates (3 µl) and lysis buffer (3 µl). Absorbance was measured at 560 nm in a microplate reader (Sunrise™, Tecan, Crailsheim, Germany) after 30 min incubation at 37 °C in a water bath.

3.4.3 Caspase activity

The activities of cysteine-dependent, aspartate-specific peptidases (caspases) were determined by using various fluorogenic substrates for caspases 2, 3, 8,

9, 10 and 12 (Table 3.7). Each substrate is comprised of a specific sequence of amino acids ending with an aspartate residue (Asp; D) labeled with the fluorogenic molecule, 7-amino-4-trifluoromethyl coumarin (AFC). The activity of individual caspase was determined by caspase cleavage of specific substrates between aspartate and AFC, thus releasing the AFC. Fluorescence of a free AFC can be quantified and is directly proportional to caspase activity.

Table 3.7: Caspase substrates used to determine specific caspase activity

Caspase	Apoptotic pathway	Substrate	Producer
2	Nuclear	VDVAD-AFC	BioVision, Milpitas, California, USA
3	Execution	DEVD-AFC	CalBioChem, Merck, Darmstadt, Germany
8	Death receptors	IETD-AFC	Enzo, Lörrach, Germany
9	Mitochondrial	LEHD-AFC	Enzo, Lörrach, Germany
10	Death receptors	AEVD-AFC	PromoKine, Heidelberg, Germany
12	Lysosomes	ATAD-AFC	PromoKine, Heidelberg, Germany

60 µl of cell lysate and 65 µl of reaction buffer (20 mM PIPES, 4 mM EDTA, 0.2 % CHAPS, 10 mM DTT, pH 7.4 and 47.3 µM caspase substrate) were pipetted into a 96-well plate. Plate was incubated at 37 °C in a water bath. The increase in fluorescence was recorded every 15 min for 1.5 h in a microplate reader (Infinite 200, Tecan, Crailsheim, Germany) at excitation/emission wavelengths of 400 nm/505 nm. The caspase activity was expressed as the change of fluorescence over one hour, normalized to the protein amount: $\Delta F/(hour \times \mu g \text{ protein})$.

3.4.4 Nuclear fragmentation

Cells were treated with OTA and ASOs as described in Section 3.2.7. After 48 h incubation, cell culture medium was removed and cells were washed once with 1× PBS. Cells were fixed with 200 µl/well of 4 % paraformaldehyde during 1 h incubation at room temperature. After washing once with 1× PBS, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) solution (5 µg/ml DAPI, 0.1 % Triton X-100, 2 mM $MgCl_2$, 100 mM PIPES, pH 6.8) for 15 min at room temperature in the dark. Afterwards, cells were rinsed with 1× PBS and analyzed by Cytation™ 3 Cell Imaging Multi-Mode Reader and automated image analysis (Gen5 software, BioTek, Bad Friedrichshall, Germany).

A maximum diameter of small nuclear fragments of 10 µm was defined in the untreated cells. Nuclear fragmentation was expressed as percentage of fragments smaller than the defined diameter, relative to the number of nuclei in the observation field.

3.4.5 Lactate dehydrogenase release

Lactate dehydrogenase (LDH) is an intracellular enzyme that catalizes the reversible conversion of pyruvate to lactate. In this reaction, co-factor NADH serves as a donor of a hydride ion and oxidizes into NAD^+.

The activity of LDH was measured in cell lysates and cell culture media. In a 96-well plate was pipetted either 2 µl cell lysate or 40 µl cell medium and reaction buffer [HEPES buffer (Table 3.8) with addition of 16.6 mM NADH and 161 mM pyruvate] up to 200 µl. Decrease in NADH absorbance at 340 nm, proportional to LDH activity, was measured during 30 min with 1 min intervals in a microplate reader (Infinite 200, Tecan, Crailsheim, Germany).

Table 3.8: Composition of HEPES buffer, pH 7.4 for LDH measurement

Reagent	Concentration (mM)
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot H_2O$)	1.0
Sodium chloride (NaCl)	122.5
Potassium chloride (KCl)	5.4
Calcium chloride dihydrate ($CaCl_2 \cdot {}_2H_2O$)	1.2
Magnesium chloride hexahydrate ($MgCl_2 \cdot {}_6H_2O$)	0.8
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	10.0

The percentage of LDH release from the cells, a measure for necrosis, was expressed as the activity of LDH in the cell medium relative to a sum of LDH activity in cell lysate and cell medium.

3.4.6 Estimation of mitochondrial membrane potential

The change in mitochondrial membrane potential was assessed by a fluorescent lipophilic cationic dye, JC-1[12] and a Mitochondrial Membrane Potential Assay Kit (Cayman chemical, Ann Arbor, USA).

In the healthy cells, high mitochondrial membrane potential (i.e. more negative than on the cytosolic side) drives the uptake of positively charged JC-1 dye into mitochondria. JC-1 concentrates in the mitochondrial matrix and allows forming J-aggregates which exhibit red fluorescence. In depolarized mitochondria with less negative potential, JC-1 exits to the cytoplasm, stays in a monomeric form and fluoresce green. This voltage-dependent spectral distinction of JC-1 dye is used to assess mitochondrial health.

Cells were treated with OTA and ASOs as described in Section 3.2.7. Cell culture media was removed and 50 µl of JC-1 dye was added per 1 ml

[12] 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-benzimidazolcarbocyanine iodide

DMEM/Ham's-F12 media. The plate was immediately placed into a Cytation™ 3 Cell Imaging Multi-Mode Reader (Gen5 software, BioTek, Bad Friedrichshall, Germany) heated to 37 °C. Fluorescence of JC-1 was measured after 10, 20 and 30 min. The red signal of J-aggregates (healthy mitochondria) was acquired at excitation/emission wavelengths of 535 nm/595 nm and the green signal of JC-1 monomers (reduced mitochondrial potential) at excitation/emission wavelengths of 485 nm/535 nm.

To indicate mitochondrial membrane potential, red to green ratio was calculated and normalized to the control/scramble ASO group. Decreased ratio reflects depolarized mitochondria.

3.5 Cellular metabolism

3.5.1 Cell culture treatment and cell lysis

Cells were treated with OTA and ASOs as described in Section 3.2.7. During 48 h, cells were incubated with different ASO and OTA combinations. To synchronize the cells after 48 h, cell culture media was removed and HEPES-Ringer buffer (Table 3.9) with 11 mM D-glucose (Sigma-Aldrich, Darmstadt, Germany) or 5 mM L-glutamine (Sigma-Aldrich, Darmstadt, Germany) was added on all cells. HEPES-Ringer buffer was incubated on cells for 3 h, then collected and used to determine glucose consumption and lactate production.

Table 3.9: Composition of HEPES-Ringer buffer, pH 7.4

Reagent	Concentration (mM)
Sodium bicarbonate ($NaHCO_3$)	24.0
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot {}_2H_2O$)	0.8
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot {}_2H_2O$)	0.2
Sodium chloride (NaCl)	86.5
Potassium chloride (KCl)	5.4
Calcium chloride dihydrate ($CaCl_2 \cdot {}_2H_2O$)	1.2
Magnesium chloride ($MgCl_2$)	0.8
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	20.0

The cell lysis procedure was carried out to collect cell lysates for the protein determination by BCA, described in Section 3.4.2. Protein concentration was, in turn, used for calibration of glucose consumption and lactate production. To lyse the cells, HEPES-Ringer buffer was collected and cells were washed with ice-cold 1× PBS. Subsequently, 100 µl/well of lysis buffer (20 mM MOPS, 0.01 % Triton-X-100, 10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5) was added. Cells were

scraped with a 1 ml-pipette tip, transferred into 1.5 ml tubes and centrifuged at 16 000× g and 4 °C for 10 min. Supernatant (i.e. cell lysate) was collected into a new 1.5 ml tube and kept on ice when BCA determination was performed immediately. Otherwise, lysates were frozen at −20 °C.

3.5.2 Glucose consumption

Glucose HK Assay Reagent (Sigma-Aldrich, Darmstadt, Germany) was reconstituted in 20 ml water. Reagent contains NAD^+ and enzymes for glucose phosphorylation and oxidation. The result of the reaction is NADH whose absorbance is directly proportional to glucose concentration.

Samples for the standard curve were prepared as 32 mM stock solution of D-glucose (Sigma-Aldrich, Darmstadt, Germany) dissolved in water and two-fold serial dilutions. For a measurement of initial glucose amount, unincubated HEPES-Ringer buffer (Table 3.9) with 11 mM D-glucose was used. HEPES-Ringer buffer incubated on the cells, unused HEPES-Ringer buffer and standards (2.5 µl of each) were pipetted into a 96-well plate and to each well 100 µl Glucose HK Assay Reagent was added.

The plate was incubated 15 min at room temperature. Absorbance was measured at 340 nm in a microplate reader (Infinite 200, Tecan, Crailsheim, Germany). Glucose consumption was calculated as the difference between initial and remaining glucose in the media, normalized per mg of protein.

3.5.3 Lactate production from D-glucose and L-glutamine

Lactate Reagent (Trinity Biotech, Wicklow, Ireland) was used to measure concentration of lactate. The reagent contains enzymes which convert lactate into pyruvate and hydrogen peroxide. The latter is involved into an activation of chromogen dye with an absorption at 540 nm. Change in absorbance is directly proportional to lactate concentration.

Samples for the standard curve were prepared as 32 mM stock solution of sodium-DL-lactate (Sigma-Aldrich, Darmstadt, Germany) dissolved in water and two-fold serial dilutions. HEPES-Ringer buffer (Table 3.9) with 11 mM glucose incubated on the cells, unused HEPES-Ringer buffer with 11 mM glucose and standards (5 µl each) were pipetted into a 96-well plate together with 50 µl Lactate Reagent. Absorbance was measured after 12 min. Lactate concentrations were expressed per mg of protein.

HEPES-Ringer buffer (Table 3.9) with 5 mM L-glutamine (without glucose)

was used to determine lactate production from other compounds than glucose, i.e. glutamine. Measurement was performed the same way as described above.

3.6 Bioinformatics tools and databases

Versions of databases and tools represent the ones that were used.

1. GENCODE database, Genome Reference Consortium Human Build 38 (GRCh38), version 30
   https://www.gencodegenes.org/human/stats.html
2. Ensembl database GRCh38, version 96
   https://www.ensembl.org/Homo_sapiens/Info/Annotation
3. NONCODE database, version 5.0
   http://www.noncode.org/analysis.php
4. LNCipedia database, version 5.2
   https://lncipedia.org/
5. lncRNAdb database, version 2.0
   https://lncrnadb.org/
6. Harmonizome database
   https://amp.pharm.mssm.edu/Harmonizome/gene/WISP
7. Primer3Plus
   https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
8. Primer-Basic Local Alignment Search Tool (BLAST)
   https://www.ncbi.nlm.nih.gov/tools/primer-blast/
9. Genome-Wide Information on Protein Synthesis VIsualiZed (GWIPS-viz)
   https://gwips.ucc.ie
10. ENCyclopedia Of DNA Elements (ENCODE) Experiment Matrix
    https://genome.ucsc.edu/encode/dataMatrix/encodeDataMatrixHuman.html
11. Integrative Genomics Viewer (IGV), version 2.3.86
    https://software.broadinstitute.org/software/igv/
12. pscan, version 1.4
    http://159.149.160.88/pscan/
13. g:Profiler, version rev. 1709
    https://biit.cs.ut.ee/gprofiler/
14. Gene Ontology enRIchment anaLysis and visuaLizAtion tool (GOrilla)
    http://cbl-gorilla.cs.technion.ac.il/

3.7 Statistical methods

When suitable, data were presented as the mean value $\pm$ standard error of the mean (SEM). Significant difference between groups was calculated by Student's *t*-test or Analysis of Variance (ANOVA) when appropriate. Calculated probability value (P) was considered significant below 0.05 ($P < .05$). n indicates number of samples per group used in the experiment. Built-in statistical tests were employed within certain programs:

- ANOVA in SigmaPlot 12.5,
- statistical test and Bonferroni correction for multiple testing in *pscan*,
- multiple testing correction in *g:Profiler* and
- statistical test in *GOrilla*.

4 Results

4.1 Molecular characteristics of *WISP1-AS1*

4.1.1 Orientation of *WISP1-AS1*

The long noncoding RNA (lncRNA) *WISP1-AS1* was discovered experimentally by a PCR method. Unfortunately, PCR cannot distinguish between the orientation of the amplified molecule. For this reason, the orientation in which this lncRNA is transcribed was unknown: sense or antisense in comparison with the protein coding *WISP1* mRNA.

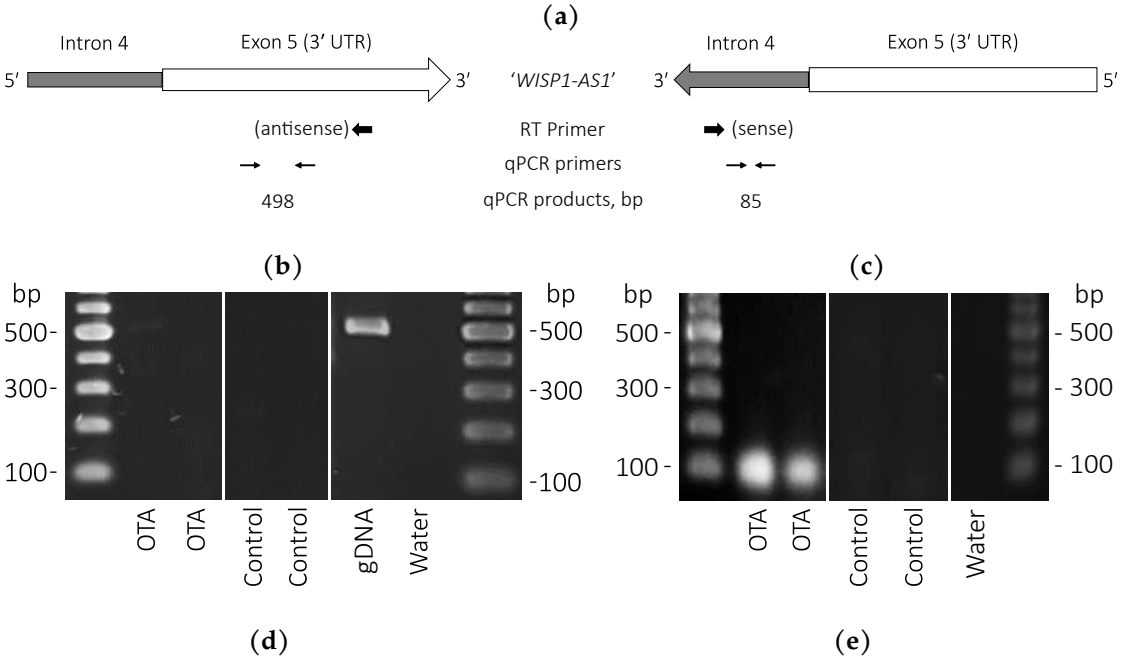
To determine *WISP1-AS1* orientation, total RNA was isolated from OTA-treated HEK293T cells and incubated with deoxyribonuclease I (DNase I). Instead of random hexamer, either sense or antisense strand-specific primer, complementary to intron 4 or 3′ UTR of *WISP1* gene, was selected for the reverse transcription (RT) reaction (Fig. 4.1a; see Table A.3 for sequences). The RT product was expected in only one reaction, corresponding to *WISP1-AS1* transcribed in antisense or sense, respectively.

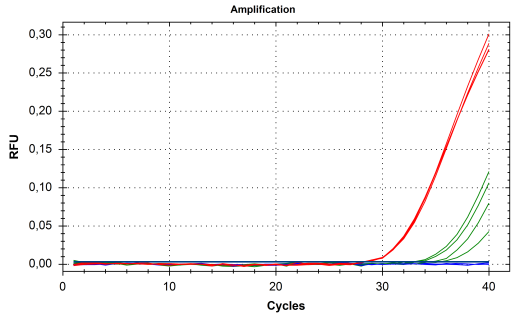
Following RT, a qPCR was performed with a set of primers (*WISP1* intron 4 (III) and *WISP1* exon 5 primer; see Table A.3 for sequences) that bind to the region downstream of the primer used in RT. By analyzing qPCR products on agarose gel, it became evident that antisense primer in RT has no complementary target molecule to reverse transcribe (Fig. 4.1b). The qPCR products were only synthesized with a sense primer in RT (Fig. 4.1c). These results mean that *WISP1-AS1* is a lncRNA transcribed in antisense, that is, in the opposite direction and off the opposite DNA strand in comparison with *WISP1* mRNA.

To ensure qPCR products were a consequence of RNA transcription and not qPCR artifacts, control qPCR reactions were included. All reactions were set without reverse transcriptase (RTase) to test for DNA contamination. To test RTase action dependent on the presence of a primer, RT reactions were performed without any primer. Both control qPCR reactions did not give an amplification (Fig. 4.1d, 4.1e), thus confirming the reliability of strand-specific RT reactions.

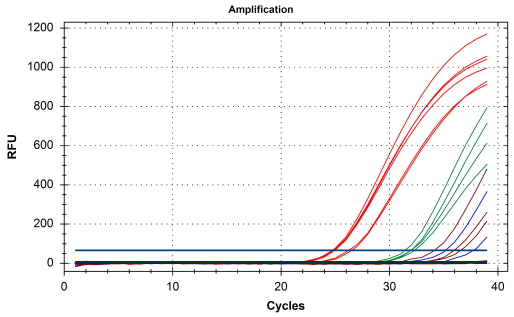
4.1.1.1 Support of *WISP1-AS1* transcription in antisense

WISP1-AS1 is a presumed lncRNA transcribed in antisense. This information is available from my studies of *WISP1-AS1* orientation by strand-specific reverse transcription, demonstrated in Fig. 4.1c and described in Section 4.1.1.





Color	Sample	RTase
Red	100 nM OTA	Yes
Green	Untreated	Yes
Blue	Untreated and 100 nM OTA	No



Color	Sample	RT Primer
Red	100 nM OTA	Yes
Green	Untreated	Yes
Blue	Untreated	No
Brown	100 nM OTA	No

Figure 4.1: Determination of *WISP1-AS1* orientation. (a) Schemes of *WISP1-AS1* in a hypothetical sense and antisense orientation. Arrows indicate positions of primers used in reverse transcription (RT) and qPCR reactions. Numbers indicate expected qPCR product sizes in base pairs (bp). (b) RT with an antisense primer, complementary to a hypothetical sense-oriented *WISP1-AS1*, did not result in a qPCR product in OTA-treated samples. qPCR primer pair is functional as shown with genomic DNA (gDNA) sample. (c) RT with a sense primer, complementary to antisense-oriented *WISP1-AS1*, resulted in qPCR products in OTA-treated samples. This demonstrates *WISP1-AS1* transcription in antisense from *WISP1* gene, in comparison with *WISP1* mRNA. (d) RNA from control (untreated) and OTA-treated cells, reverse transcribed with and without RTase. Reactions without RTase, shown in blue, confirmed good DNase I treatment. (e) RT with and without primers confirm specific primer-dependent RTase activity.

The following analysis employed an *in silico* database to support these results and to seek for an evidence of antisense transcription from *WISP1* locus. The database, the encyclopedia of DNA elements (ENCODE) Experiment Matrix, is

a 2D array of different cell types and different genome-wide experiments.

By browsing the ENCODE Experiment Matrix, I found that certain RNA-seq experiments, performed with normal human epidermal keratinocyte (NHEK) cells, showed an antisense transcription from *WISP1* locus (Fig. 4.2). Transcription was evident only on a "minus" strand and not on a "plus" strand, indicating orientation-specific transcript. These data confirmed that *WISP1-AS1* transcription in antisense is possible and present in some cell types.

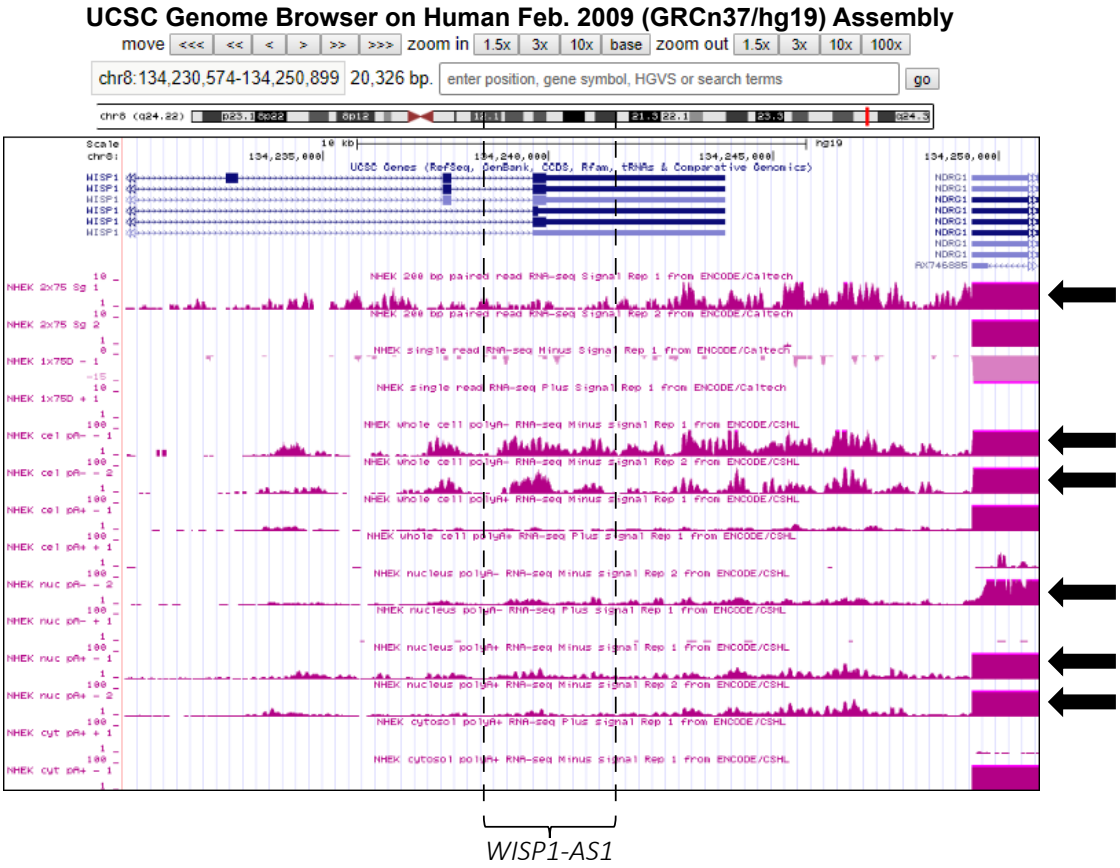


Figure 4.2: *In silico* analysis of RNA-seq experiments. Normal human epidermal keratinocytes (NHEK) show an evidence of an antisense transcription from *WISP1* locus that corresponds to *WISP1-AS1*. Transcription is observed in some RNA-seq experiments only on a "minus" strand (indicated by arrows) and not on a "plus" strand, showing orientation specificity. Last downloaded and modified on 10 July 2018. On the https://genome.ucsc.edu/encode/dataMatrix/encodeDataMatrixHuman.html choose a matrix between "NHEK" cells on the left and "RNA-seq" on the top. In the new window that opens, click on the boxes that contain RNA-seq on the minus strands, e.g. "NHEK whole cell polyA+ RNA-seq Minus signal Rep 1 from ENCODE/CSHL". Choose "dense" view. Click on "view in browser" button.

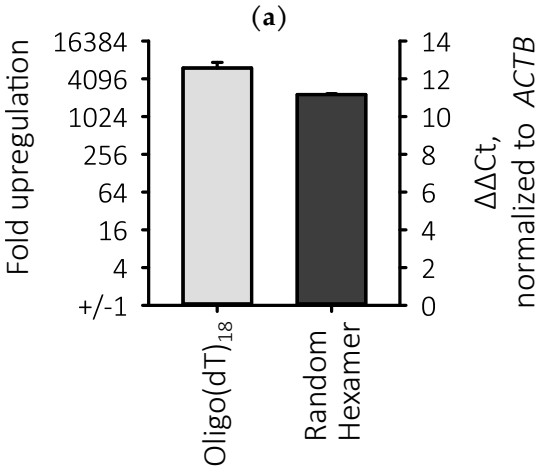
4.1.2 Polyadenylation of *WISP1-AS1*

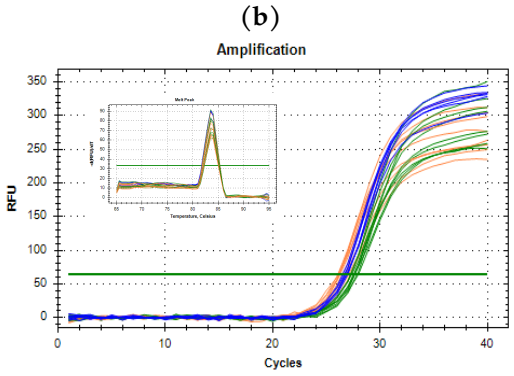
To determine whether lncRNA *WISP1-AS1* is polyadenylated, total RNA from 100 nM OTA-treated HEK293T cells was DNase I-treated, then reverse

transcribed with oligo$(dT)_{18}$ primer and, in parallel, with random hexamers.

A subsequent qPCR reaction was performed with *WISP1* intron 4 (IV) primer pair (see Table A.3 for the sequences). When *WISP1-AS1* expression was compared between two different reverse transcription approaches, similar upregulation was observed (Fig. 4.3a). A signal in oligo$(dT)_{18}$ reactions verified *WISP1-AS1* polyadenylation.

Another approach to evaluate polyadenylation was to isolate the polyadenylated fraction of RNA and analyze whether it contained *WISP1-AS1*. Figure 4.3b confirms *WISP1-AS1* polyadenylation in OTA-treated cells, in RT reactions with oligo$(dT)_{18}$ and with random hexamers. qPCR reactions were performed with *WISP1* intron 4 (V) primer pair (see Table A.3 for the sequences).





Color	RNA used in RT	RT Primer
Orange	Total	Random
Green	Total	Oligo$(dT)_{18}$
Blue	Polyadenylated	Oligo$(dT)_{18}$

Figure 4.3: *WISP1-AS1* polyadenylation. (a) Total RNA from OTA-treated HEK293T cells was reverse transcribed with oligo$(dT)_{18}$ or random hexamer and then amplified with PCR primers in *WISP1* intron 4. *WISP1-AS1* is strongly upregulated in both reactions, confirming *WISP1-AS1* polyadenylation. Expression compared to *ACTB*. Data are shown as means ± SEM. n = 3. (b) Comparison between OTA-treated total RNA, reverse transcribed with random hexamer and oligo$(dT)_{18}$, and polyadenylated RNA, reverse transcribed with oligo$(dT)_{18}$. qPCR results show that *WISP1-AS1* appears in all reactions, thus confirming *WISP1-AS1* polyadenylation. Insert shows melting curves. n = 6 (total RNA), n = 9 (polyadenylated RNA).

4.1.3 Transcriptional landscape of *WISP1-AS1*

4.1.3.1 Influence of *WISP1-AS1* on *WISP1* mRNA

WISP1-AS1 is transcribed from the opposite strand of DNA compared to *WISP1* mRNA and, therefore, between lncRNA and mRNA exist sequence complemen-

tarity. Because of *WISP1-AS1* orientation in antisense, it is possible that it influences *WISP1* mRNA.

To clarify this probability, first I analyzed untreated HEK293T cells for cotranscription of *WISP1-AS1* and *WISP1* mRNA. For this purpose, TaqMan probes specific for *WISP1* mRNA and *WISP1-AS1* were designed (see Table A.3 for sequences). In untreated cells, *WISP1-AS1* expression was low (Ct values $\geq$ 38) and *WISP1* mRNA was untraceable (Fig. 4.4a). However, in OTA-induced cells, only *WISP1-AS1* showed an upregulation, while no amplification of *WISP1* mRNA was observed.

These data demonstrated that HEK293T cells do not express *WISP1* mRNA, not even after OTA treatment. However, how to be sure that *WISP1-AS1* does not suppress *WISP1* mRNA in OTA-treated cells? Is the absence of the signal in PCR with TaqMan primers for *WISP1* mRNA:

1. due to no *WISP1* mRNA expression in OTA-treated HEK293T cells?
2. an indicator of the suppression by *WISP1-AS1*?

To address the first question, RNA from OTA-treated cells was analyzed again for *WISP1* mRNA expression. A qPCR carried out with *WISP1* mRNA-specific primers (exon 1 – exon 2 primers spanning the first intron of *WISP1* gene; see Table A.3 for sequences) show slight downregulation of *WISP1* mRNA in OTA-treated cells (Fig. 4.4b). The results confirm the functionality of TaqMan primers specific for *WISP1* mRNA used in Figure 4.4a, which correctly show no amplification of *WISP1* mRNA due to the lack of *WISP1* mRNA expression in OTA-treated cells.

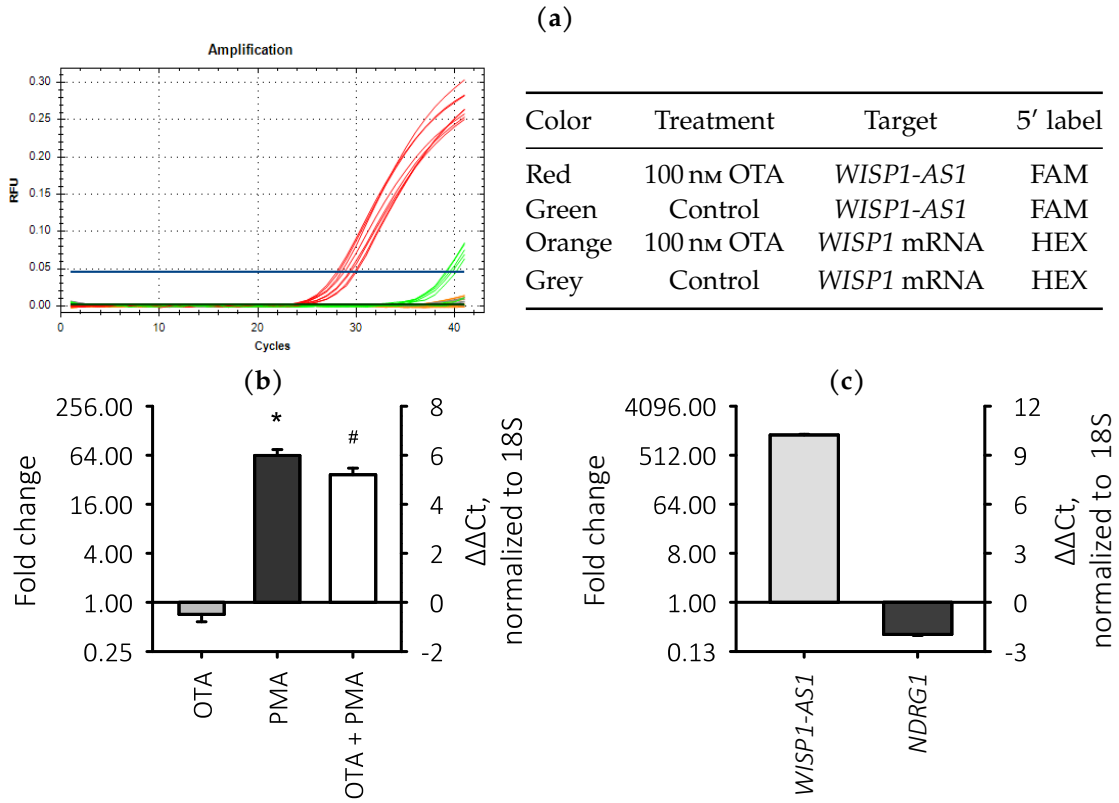
As a positive control, cells were treated with PMA to induce *WISP1* mRNA transcription (see Section 1.8 for description of this mechanism). When treated with PMA, qPCR performed with primers specific for *WISP1* mRNA (exon 1–exon 2 primers; see Table A.3 for sequences) show *WISP1* mRNA upregulation (Fig. 4.4b).

To focus on the second question, cells were treated simultaneously with OTA and PMA to induce both transcripts. If *WISP1-AS1* suppresses the expression of *WISP1* mRNA, then, when both transcripts are present, *WISP1* mRNA expression should be strongly reduced. This would confirm that *WISP1-AS1* suppresses *WISP1* mRNA.

In Figure 4.4b, very small reduction in *WISP1* mRNA expression occurred when both transcripts were induced. *WISP1* mRNA expression was not abolished, indicating that it might be *WISP1-AS1* independent.

Taken together, these data show that *WISP1-AS1* and *WISP1* mRNA have

different inducers in HEK293T cells: OTA induces only *WISP1-AS1* (Fig. 4.4a) and PMA induces only mRNA (Fig. 4.4b). In conclusion, I assume that *WISP1-AS1* does not suppress *WISP1* mRNA because mRNA expression is still high in OTA+PMA-treated cells when both transcripts are simultaneously present.



Color	Treatment	Target	5′ label
Red	100 nM OTA	*WISP1-AS1*	FAM
Green	Control	*WISP1-AS1*	FAM
Orange	100 nM OTA	*WISP1* mRNA	HEX
Grey	Control	*WISP1* mRNA	HEX

Figure 4.4: Expression of *WISP1-AS1*, *WISP1* mRNA and *NDRG1* mRNA. (a) TaqMan probes, designed specifically for *WISP1-AS1* or *WISP1* mRNA, show that in control (untreated) cells, *WISP1-AS1* expression is low and in OTA-treated cells is upregulated. There is no *WISP1* mRNA expression in neither control nor OTA-treated cells. n = 6. (b) *WISP1* mRNA expression. qPCR performed with *WISP1* mRNA-specific primers (*WISP1* exon 1 - exon 2 primer pair). OTA leads to a slight downregulation of *WISP1* mRNA. PMA was used as a positive control to induce *WISP1* mRNA. *WISP1* mRNA expression is significantly upregulated by PMA when compared to control cells. To test for *WISP1* mRNA suppression by *WISP1-AS1*, both transcripts were induced by OTA + PMA. *WISP1* mRNA expression was slightly but significantly reduced. However, mRNA expression was not completely abolished, indicating *WISP1-AS1* independence. Data are shown as means ± SEM. Asterisk and hashtag indicate significant difference ($P < .05$) from the control and PMA group, respectively. n = 9. (c) In the conditions when *WISP1-AS1* is upregulated, the expression of *NDRG1* is slightly repressed. These data indicate two separate transcripts, *WISP1-AS1* and *NDRG1* mRNA, otherwise their expression would be in concordance. Data are shown as means ± SEM. n = 4.

4.1.3.2 Transcriptional elongation from the upstream gene

Since *WISP1-AS1* is in antisense orientation to *WISP1* mRNA and it probably has no suppressive function on *WISP1* mRNA, another critical feature to test was whether *WISP1-AS1* transcription belongs to an upstream gene. In general, two genes show similar expression pattern if they are coregulated/coexpressed.

In the 5′ direction of *WISP1-AS1* is the N-myc downstream regulated gene 1 (*NDRG1*), involved in differentiation, caspase activation and apoptosis. The distance between the end of *NDRG1* gene and the beginning of *WISP1-AS1* is 7750 bp, according to NCBI Genome Data Viewer[1].

WISP1-AS1 and *NDRG1* expression was analyzed by qPCR in total RNA from OTA-treated cells with *WISP1* intron (IV) and *NDRG1* primers (see Table A.3 for sequences). When *WISP1-AS1* was upregulated by OTA, *NDRG1* mRNA expression was slightly downregulated (Fig. 4.4c), showing independent transcription of the two adjacent genes. In other words, *WISP1-AS1* is not a transcriptional continuation of the upstream gene, *NDRG1*. This in turn supports the existence of a lncRNA, *WISP1-AS1*, as a separate transcript.

4.1.4 Noncoding potential of *WISP1-AS1*

All analyzed *in silico* lncRNA databases did not predict an existence of a noncoding transcript in *WISP1* locus, in the intron 4, exon 5 and 3′ UTR from where *WISP1-AS1* is transcribed. Without an evidence of its existence, a question arose: Is *WISP1-AS1* really a noncoding RNA molecule or does it have the potential to translate into peptides? To answer this question, an online database of translation, genome-wide information on protein synthesis visualized (GWIPS-viz) was employed, described by [MICHEL ET AL. 2013]. This database consists of 44[2] ribosome profiling experiments from different studies on different cell types.

I browsed the sequence of *WISP1* gene in all the published studies in GWIPS-viz and observed translational events only in the regions of exons (Fig. 4.5). No translation in intron 4 nor 3′ UTR was visible from where *WISP1-AS1* is transcribed.

The downside of the database is that it does not contain cells treated with OTA, the only known inducer of *WISP1-AS1*. From this database information, I can only assume there is no translation of *WISP1-AS1*, at least not in the analyzed cells. In that case, *WISP1-AS1* should function solely as RNA molecule.

[1] https://www.ncbi.nlm.nih.gov/genome/gdv/browser/?context=gene&acc=10397
[2] On 13 December 2019

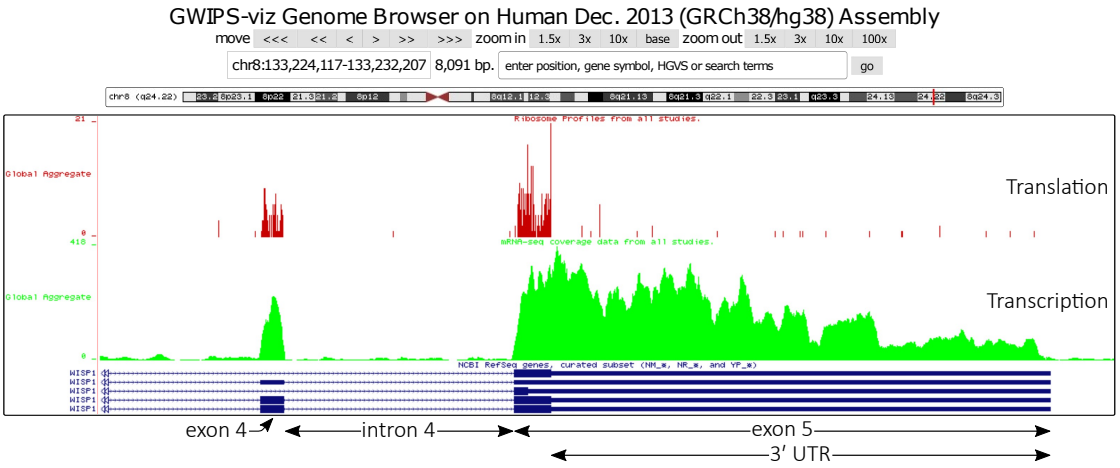


Figure 4.5: *In silico* ribosome profiling. Translational events are shown in red in the upper part of the panel. Besides translation of exons 4 and 5 that belongs to *WISP1* mRNA, there is no substantial translation in intron 4 nor in 3′ UTR from where *WISP1-AS1* is transcribed. These data support noncoding feature of *WISP1-AS1*. Last downloaded and modified on 13 December 2019 from: https://gwips.ucc.ie/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr8%3A133224117-133232207&hgsid=53858_QXJ74F9Fr6YEETdobe4ec7GFB3q4

4.2 *WISP1-AS1* expression

4.2.1 *WISP1-AS1* expression in renal cancer cells

OTA is suspected to contribute to the incidence of renal and urinary tract tumors in humans (KUIPER-GOODMAN AND SCOTT 1989). Moreover, many lncRNAs were found to be differentially expressed in human clear cell renal cell carcinoma samples (BLONDEAU ET AL. 2015). OTA treatment of renal cells causes an upregulation of *WISP1-AS1*, as I demonstrated in my results (Fig. 4.3a and Section 4.1.3.1). Therefore, in case of *WISP1-AS1*, it was of importance to address the following questions:

1. Is *WISP1-AS1* expressed at all in renal *cancer* cells and, if yes, to what extent?
2. Does OTA lead to (further) *WISP1-AS1* upregulation in renal *cancer* cells?

To clarify the expression pattern of the novel transcript *WISP1-AS1* in renal cancer cells, I analyzed clear cell renal cell carcinoma (ccRCC) cells, 786-O, derived from the epithelium of the renal proximal tubule. Droplet digital PCR (ddPCR) was used to quantify the copy number of *WISP1-AS1* per 1 µg of total RNA used. DdPCR was performed with *WISP1* intron (IV) primer pair (see Table A.3 for the sequences).

In the 786-O cell line, *WISP1-AS1* expression was already elevated under basal conditions (Fig. 4.6), compared with untreated HEK293T cells where copy number of *WISP1-AS1* per 1 µg of total RNA was between 0 and 500 (data not shown). To analyze OTA ability to further induce *WISP1-AS1* expression in cancer cells, 786-O cells were treated with 100 nM OTA for 48 h. Results showed tremendous *WISP1-AS1* upregulation: *WISP1-AS1* copy number increased 13 times, compared with untreated 786-O cells.

High *WISP1-AS1* expression in the renal cancer cells without induction by OTA points out the potential involvement of *WISP1-AS1* in carcinogenesis.

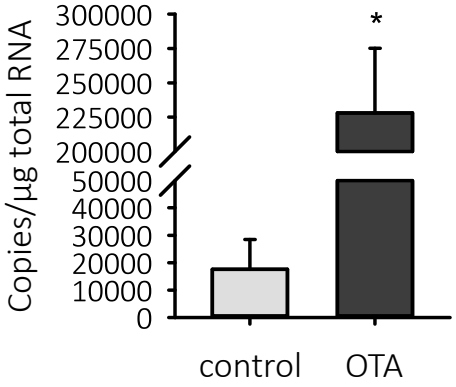


Figure 4.6: *WISP1-AS1* expression in ccRCC cell line 786-O. Total RNA from untreated (control) and 100 nM OTA-treated ccRCC 786-O was analyzed by ddPCR for *WISP1-AS1* expression. In untreated cells, *WISP1-AS1* copy number was already high and it was tremendously upregulated by OTA induction.
Data are shown as means ± SEM. Asterisk indicates significant difference ($P < .05$) from the control group. n = 9 (controls), n = 14 (OTA).

4.2.2 Northern blot confirmation of *WISP1-AS1* expression and orientation

LncRNAs are often low-abundant transcripts. For that reason, ccRCC 786-O cells were used for Northern blot, since OTA-induced *WISP1-AS1* level was much higher in these cells than in induced HEK293T cells. Therefore, total RNA, isolated from OTA-treated or untreated ccRCC 786-O cells, was separated on urea-agarose gel and transferred on a nylon membrane.

Two biotinylated probes were produced: a probe in antisense to *WISP1-AS1* served to detect *WISP1-AS1*, and a probe in sense was a negative control to show sequence- and orientation-specificity of the antisense probe. A biotinylated probe directed against *WISP1-AS1* detected a single band at around 3000 nt in 786-O cells treated with OTA (Fig. 4.7a). The band corresponds to *WISP1-AS1* size of 2922 nt, confirming the *WISP1-AS1* expression. Additionally, results confirm *WISP1-AS1* orientation in antisense in comparison with *WISP1* mRNA.

A negative control probe - a probe with a sequence identical to *WISP1-AS1* - did not show any bands (Fig. 4.7b). The lack of signal is consistent with the hypothesis that *WISP1-AS1* expresses only in antisense direction to *WISP1* mRNA.

Both membranes were stripped and re-probed with a biotinylated probe for *ACTB* mRNA, an internal standard. In both cases, a clear band at 1700 nt was

visible (Fig. 4.7a,4.7b, lower panels).

Hybridization control reactions were performed with a biotinylated probe directed against *WISP1-AS1* and a negative control probe. The results show the ability of both probes to specifically recognize only the complementary target (Fig. 4.7c).

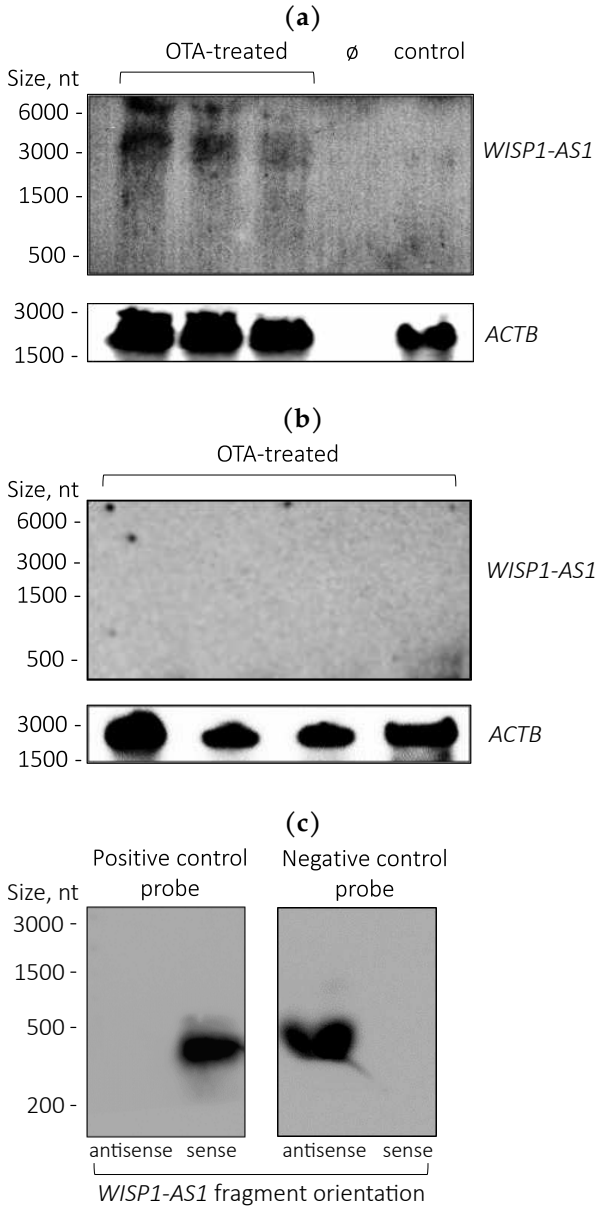


Figure 4.7: Northern blot. RNA from OTA-treated and control 786-O cells was separated on urea-agarose gel and transferred on a nylon membrane. Each OTA-treated line is RNA from an individual Petri dish of 786-O cells. **(a)** Biotinylated probe directed against *WISP1-AS1* recognized a target in ccRCC 786-O cells treated with 100 nM OTA for 48 h. The band size corresponds to 2922 nt (nucleotides) of *WISP1-AS1*. **(b)** Biotinylated probe directed against complementary sequence of *WISP1-AS1* (negative control probe) did not recognize the target. Internal control, *ACTB* mRNA, shows bands of 1700 nt in **(a)** and **(b)**. **(c)** Hybridization specificity of biotinylated probes. Samples loaded on the gel are *in vitro* transcribed 364 nt sense or antisense fragments of *WISP1-AS1*. Positive control probe recognizes *WISP1-AS1* (i.e. sense-oriented fragment, in 5′ to 3′ direction of *WISP1-AS1*). Negative control probe recognizes a sequence complementary to *WISP1-AS1* (i.e. antisense-oriented fragment, in 3′ to 5′ direction of *WISP1-AS1*). Each biotinylated probe hybridizes only to its complementary fragment, confirming the ability to specifically recognize the target.

4.3 Knockdown tool for *WISP1-AS1* functional studies

To get an insight into *WISP1-AS1* functions, I studied the cellular consequences in conditions of inhibited *WISP1-AS1* expression.

For that purpose, LNA™ GapmeR antisense oligonucleotides (ASOs) were

designed, complementary to intron 4 part of *WISP1-AS1*. I tested ASOs in cell culture and established a knockdown method to reduce *WISP1-AS1* expression. Subsequently, HEK293T cells were treated by OTA to induce *WISP1-AS1*, and transfected by ASOs to knockdown *WISP1-AS1*.

Analyzed by qPCR, knockdown by ASOs in the cells treated with OTA reduced *WISP1-AS1* copy number eight-fold in comparison with OTA-treated cells in the presence of scramble ASO (Fig. 4.8a). WISP1 intron (IV) primers were used (see Table A.3 for the sequences).

To show orientation specificity of ASOs on the knockdown outcome, sense ASOs were designed, with a sequence identical to *WISP1-AS1*. As shown in Figure 4.8b, sense ASOs did not knockdown *WISP1-AS1*; only antisense ASOs reduced *WISP1-AS1* expression down to 10 %.

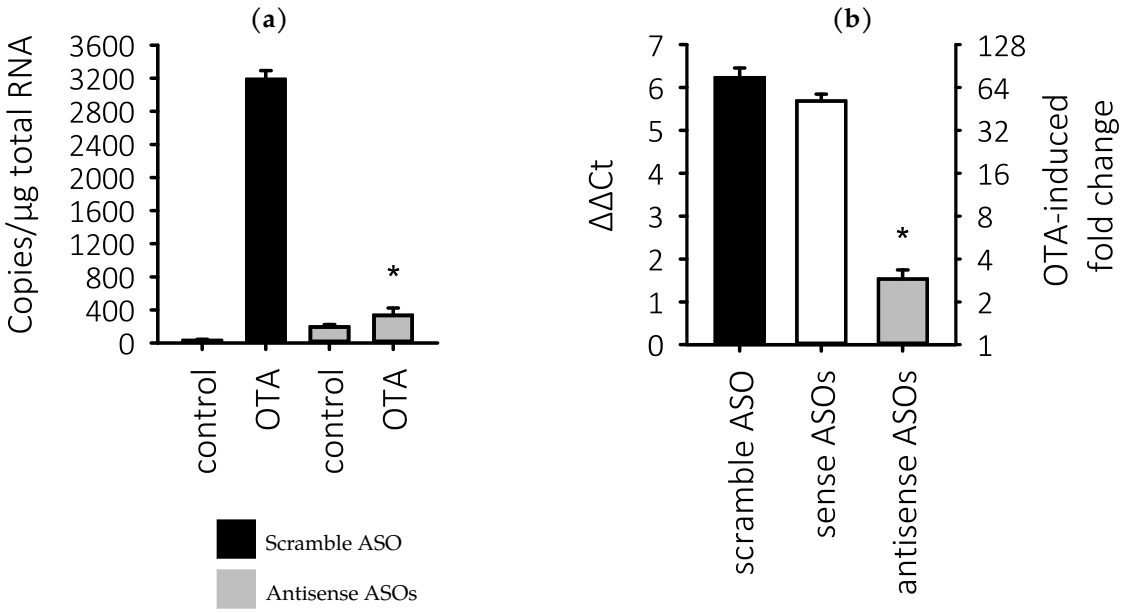


Figure 4.8: OTA-induced *WISP1-AS1* expression in the presence of LNA GapmeR antisense oligonucleotides (ASOs). (**a**) ASOs complementary to *WISP1-AS1* (antisense ASOs) reduced *WISP1-AS1* expression in OTA-treated cells (the OTA/antisense ASO group), analyzed by ddPCR. Data are shown as means ± SEM. Asterisk indicates significant difference ($P < .05$) from the OTA/scramble ASO group. n = 6. (**b**) The effect of scramble ASO, ASOs in the same orientation as *WISP1-AS1* (sense ASOs) and ASOs complementary to *WISP1-AS1* (antisense ASOs) on *WISP1-AS1* expression in OTA-treated cells, analyzed by qPCR. Only antisense ASOs knockdown *WISP1-AS1*. Data are shown as means ± SEM. Asterisk indicates significant difference ($P < .05$) from scramble ASO. n = 4.

4.4 RNA-sequencing

4.4.1 Differential expression of protein coding genes

To discover the impact of *WISP1-AS1* on gene expression regulation, whole transcriptomes were analyzed for changes caused by OTA-induced *WISP1-AS1*. For that, RNA from four groups of samples (described in Table 3.4) was subjected to RNA-seq:

1. OTA/scramble ASO,
2. OTA/*WISP1-AS1* ASOs,
3. control/scramble ASO,
4. control/*WISP1-AS1* ASOs.

RNA-seq resulted in over 58 000 transcripts separated in five groups of expression: 'protein coding' (20 328), 'lincRNAs' (7110), 'antisense' (5274), 'microRNAs' (3050) and 'others' (22 016). To unveil a mechanism of the function of *WISP1-AS1*, only 'protein coding' transcripts were included for further analysis.

To observe changes in gene expression that are absolutely dependent on *WISP1-AS1*, differential expression (DE) was performed on the protein coding transcripts as shown in Figure 4.9a. Only OTA-induced, *WISP1-AS1*-dependent genes were selected when up- or down-regulated in the OTA/scramble ASO group, in comparison with the control/scramble ASO group. DE between other groups should have shown no significant change, illustrated by DE = 0.

OTA-induced, *WISP1-AS1*-dependent genes were grouped in a 2D array by fold change (FC) and fragments per million (FPM) (Fig. 4.9b,4.9c). When sorted that way, 617 upregulated and 758 downregulated genes were significantly changed. For the upregulated genes, FPM was taken from the OTA/scramble ASO group. In case of downregulated genes, FPM of the control/scramble ASO group was used. This means that, for example, in upregulated genes, a gene in the *OTA/scramble ASO* group shows FPM > 20 (while the same gene in the control/scramble ASO group shows FPM < 20). In case of downregulated genes it is the opposite: a gene in the *control/scramble ASO* shows FPM > 20 (while in the OTA/scramble ASO group, the same gene shows FPM < 20).

One gene that is upregulated with FC > 3 and FPM between 20–100 is *THSD4* (thrombospondin type 1 domain containing 4) gene (Fig. 4.9b). It is also known as *ADAMTSL-6* (a disintegrin and metalloproteinase with thrombospondin motifs-like protein 6) gene. THSD4 is described to promote

extracellular matrix, in particular, fibrillin-1 matrix assembly [Tsutsui2010]. The only gene downregulated with FC < 4 and FPM between 10–150 is *NDUFB10* (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10) gene (Fig. 4.9c). NDUFB10 is an accessory subunit of the enzyme NADH dehydrogenase (ubiquinone) complex in the inner mitochondrial membrane, also known as Complex I.

Up- or down-regulated genes were further analyzed for the enrichment of gene ontology (GO) terms and TFBSs within their promoter regions.

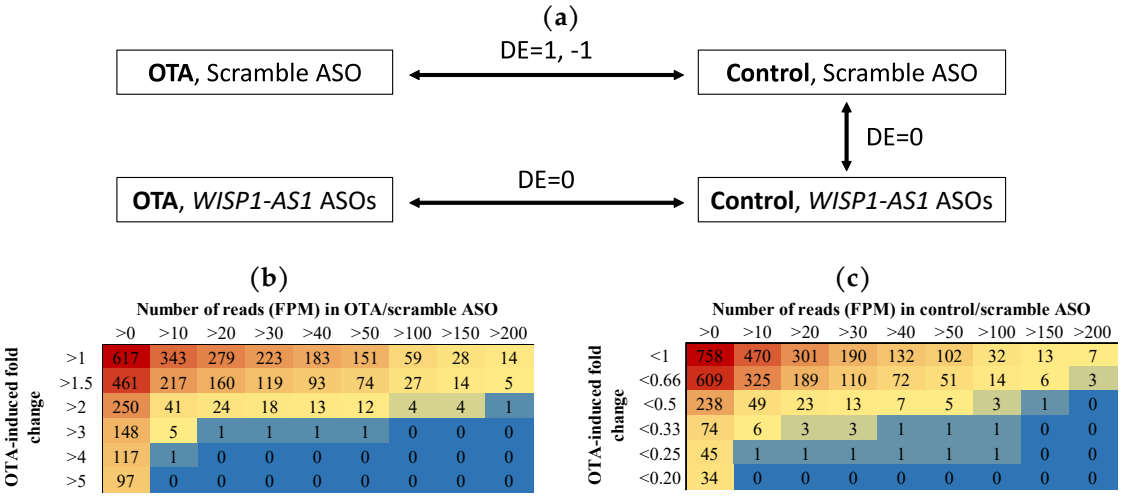


Figure 4.9: Differential expression (DE) analysis of RNA-seq genes. (a) To filter the genes that are absolutely dependent on *WISP1-AS1*, comparison of DE was performed between the groups indicated on the scheme. Changes in gene expression were allowed only between the OTA/scramble ASO and the control/scramble ASO group. DE $= 1$ means upregulation in the OTA/scramble ASO group; DE $= -1$ means downregulation in the OTA/scramble ASO group; DE $= 0$ means no changes between the two depicted groups. (b) Upregulated genes and (c) downregulated genes, sorted by OTA-induced FC and number of reads, i.e. fragments per million (FPM) in the OTA/scramble ASO and the control/scramble ASO group, respectively.

4.4.2 Gene ontology enrichment analysis

One way to analyze large gene lists and observe a pattern which might explain the function of *WISP1-AS1* is to look for enrichment in GO terms. GO categories in *g:Profiler* and *GOrilla* are: 'biological process', 'cellular component' and 'molecular function'. Each category contains terms, e.g. molecular pathway, organelle, enzyme activity, etc.

The GO enrichment analysis was conducted with OTA-induced, *WISP1-AS1*-dependent genes. Up- or down-regulated genes were chosen with FC $> |1.5|$ and FPM > 10 in the OTA/scramble ASO or the control/scramble ASO group,

respectively.

With these parameters, 217 genes appeared upregulated but *g:Profiler* and *GOrilla* did not recognize any significantly enriched gene cluster within any GO category. Downregulated 325 genes appeared enriched in *GOrilla* in 'cellular component - organelle part - nucleolus', whereas *g:Profiler* did not result in enrichment. Nucleolus enrichment was no more significant in the genes downregulated with FC < 1.5 and FPM > 20 in the control/scramble ASO group.

From the results, I conclude that *WISP1-AS1* probably does not influence any gene cluster. Its function might be more specific, like influencing one or a few coding or regulatory RNAs by various mechanisms. Therefore, the following analysis was performed.

4.4.3 *WISP1-AS1* influence on transcription factor activity

Another way to find common elements between genes changed by *WISP1-AS1* is to seek for common response elements in their promoters. Every gene is controlled by one or more response elements in the promoter region. Response elements, i.e. TFBSs are recognized by specific transcription factors (TFs). Generally speaking, TFBSs can appear enriched within the promoters, indicating that some cell treatment might had affected those TFs and resulted in the observed gene expression.

OTA-induced, *WISP1-AS1*-dependent genes chosen for the analysis contained the same parameters as in the previous section: FC $> |1.5|$ and FPM > 10 in the OTA/scramble ASO or the control/scramble ASO group.

Analyzed by *g:Profiler*, more than 70 % of upregulated genes in their promoter regions contained overrepresented TFBSs for TFs: ETF, E2F, ZF5, SP1, WT1 and EGR-1 ($10^{-7} > P > 10^{-14}$). A *pscan* analysis of promoter regions confirmed enrichment of EGR-1, SP1 and E2F (-950 to $+50$ bp from transcription start site (TSS) ($10^{-7} > P > 10^{-10}$).

For downregulated genes, *g:Profiler* analysis resulted in a similar fashion. More than 70 % genes contained overrepresented TFBSs for TFs: E2F, ETF, ZF5, ELK-1, EGR-1 and SP1 ($10^{-8} > P > 10^{-20}$). Four of them were confirmed by *pscan*: SP1, ELK-1, E2F and EGR-1 (-950 to $+50$ from TSS) ($10^{-8} > P > 10^{-12}$).

The finding that TFBSs appeared overrepresented in a *WISP1-AS1*-dependent manner suggests *WISP1-AS1* function as a modulator of TF activity. Possibly, *WISP1-AS1*-induced changes in TFs regulated gene expression and resulted in up- or down-regulated transcripts observed in RNA-seq.

4.4.4 Testing the TF activity by reporter assays

The activities of some TFs, whose TFBSs were *in silico* predicted to be overrepresented in differentially expressed genes, were further analyzed by reporter assays.

To acquire OTA influence on the activity of TFs, HEK293T cells were transfected with a vector carrying response element for TF EGR-1, E2F or AP-1. Afterwards, cells were treated with different concentrations of OTA (Fig. 4.10a, 4.10b,4.10c). To evaluate *WISP1-AS1* involvement in OTA-induced changes in TF activity, HEK293T cells were transfected with vectors carrying EGR-1 or E2F and then treated with ASOs (scramble or *WISP1-AS1* ASOs) and 100 nM OTA (Fig. 4.10d,4.10e). The activities of TFs were measured 24 h after OTA addition in all cases. EGR-1 activity was measured by Cytation™ 3 Cell Imaging Multi-Mode Reader and E2F activity was measured by the Dual-Luciferase® Reporter (DLR™) assay. See Section 3.3.2 for detailed treatment.

To investigate the influence of OTA on the activity of TFs, cells were transfected with vectors carrying cloned response elements for E2F or EGR-1 and then incubated with different concentrations of OTA. After 24 h, EGR-1 activity was significantly *increased* by OTA concentrations above 10 nM (Fig. 4.10a). In contrast, the activity of E2F was significantly *decreased* by OTA concentrations above 10 nM (Fig. 4.10b).

The analyzed TFs are ubiquitous regulators of gene expression. To evaluate whether changes in overrepresented TFBSs could show up by accident in the analysis, another ubiquitous TF, AP-1, was used as a test case. AP-1 did not appear enriched in gene sets analyzed by *g:Profiler* and *pscan*. In reporter assay experiments, different concentrations of OTA during 24 h did not affect AP-1 activity (Fig. 4.10c). This analysis confirmed that TFBSs, identified by *g:Profiler* and *pscan*, correspond to OTA-induced changes. In other words, OTA does not affect all promoters in an unspecific way but rather acts only on certain promoters by changing the activity of some TFs.

To determine how the TF activity can be modulated by the presence or absence of *WISP1-AS1*, GapmeR ASOs were employed to knockdown *WISP1-AS1*. EGR-1 activity was increased by OTA-induced *WISP1-AS1* (Fig. 4.10d). When *WISP1-AS1* was knocked down by ASOs, EGR-1 activity was significantly lower. On the other hand, OTA-induced *WISP1-AS1* suppressed E2F activity (Fig. 4.10e). In the absence of *WISP1-AS1*, suppression of E2F activity was weaker.

Both cases exemplify a potential mechanism of *WISP1-AS1* as a modulator

of TF availability to the target's promoter regions which, in turn, regulates gene expression.

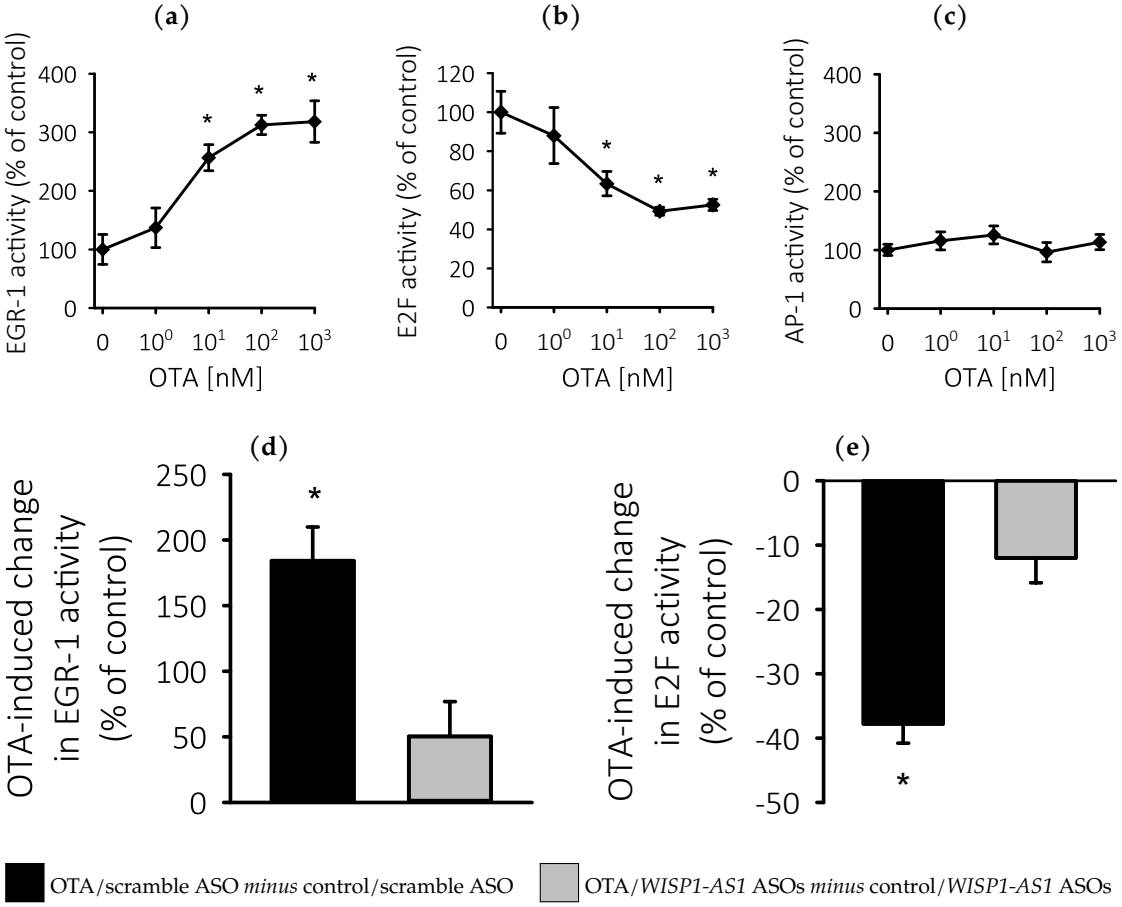


Figure 4.10: OTA and *WISP1-AS1* influence on the transcriptional activity of EGR-1, E2F and AP-1. **(a)** EGR-1 activity was significantly induced after 24 h incubation with 10, 100 and 1000 nM OTA. **(b)** E2F activity was significantly reduced after 24 h incubation with 10, 100 and 1000 nM OTA. **(c)** AP-1 activity was not changed by OTA during 24 h. In **(a)**, **(b)** and **(c)**, asterisk indicates significant difference ($P < .05$) from control (0 nM OTA). **(d)** EGR-1 activity was increased in the presence of *WISP1-AS1* (i.e. in the presence of the scramble ASO). When *WISP1-AS1* was knocked down by ASOs, EGR-1 activity was decreased. **(e)** The activity of E2F was repressed in the presence of *WISP1-AS1*. *WISP1-AS1* knockdown by ASOs resulted in weaker repression of E2F activity. In **(d)** and **(e)**, asterisk indicates significant influence of OTA ($P < .05$). Data in all graphs are shown as means $\pm$ SEM. n = 12 in all graphs.

4.4.5 Gene expression validation

Following RNA-seq data analysis, experimental validation of up- and down-regulated genes was performed by qPCR and ddPCR. Validation by other approaches is necessary to increase the confidence in RNA-seq results.

Genes chosen for the validation contained FC > |1.5| and FPM > 20 in the OTA/scramble ASO group (for upregulated genes) or the control/scramble

ASO group (for downregulated genes).

In 28 analyzed genes, up- or down-regulation was confirmed between the OTA/scramble ASO and the control/scramble ASO group (Fig. 4.11a). Validation was successful in 93 % of cases where genes have shown the same direction in expression in qPCR and ddPCR as in RNA-seq. See Table A.3 for the primer sequences.

THSD4 and *NDUFB10* were validated too, the two genes that appeared changed persistently with an increasing FC and FPM in a 2D array of RNA-seq results. Only *SIRT1* and *RPL39* have shown the opposite direction in qPCR, compared with RNA-seq. Validation demonstrates that differential expression of genes in RNA-seq can be taken reliably.

One target gene, the growth-arrest specific gene 6 (*GAS6*) is shown here in detail. *GAS6* mRNA was upregulated in the presence of OTA-induced *WISP1-AS1*, in RNA-seq results (Fig. 4.11b) and confirmed by ddPCR (Fig. 4.11c). In the conditions when *WISP1-AS1* was knocked down by ASOs, *GAS6* expression was reduced.

4.5 Cell death

4.5.1 *WISP1-AS1* involvement in apoptosis

To analyze whether OTA-induced *WISP1-AS1* is involved in apoptotic changes at low OTA concentrations of 100 nM, caspase-3 activity was measured by using florigenic substrate. Caspase-3 is an executioner caspase in the apoptotic pathway.

With this assay, OTA-induced caspase-3 activity was low when *WISP1-AS1* was present in cells, i.e. when cells were treated with OTA/scramble ASO (Fig. 4.12a). In contrast, high OTA-induced caspase-3 activity was assessed when *WISP1-AS1* expression was decreased, i.e. in OTA-treated cells with ASOs to knockdown *WISP1-AS1*. In these cells, caspase-3 activity tripled in comparison with the control/scramble ASO group, representing increased apoptosis.

These findings suggest a protective role of *WISP1-AS1* against apoptosis.

Different apoptotic pathways can trigger caspase-3 activation. To asses which upstream caspase is involved, activities of caspases 2, 8, 9, 10 and 12 were measured.

Caspase-2, an initiator caspase of nuclear apoptotic pathway, showed in-

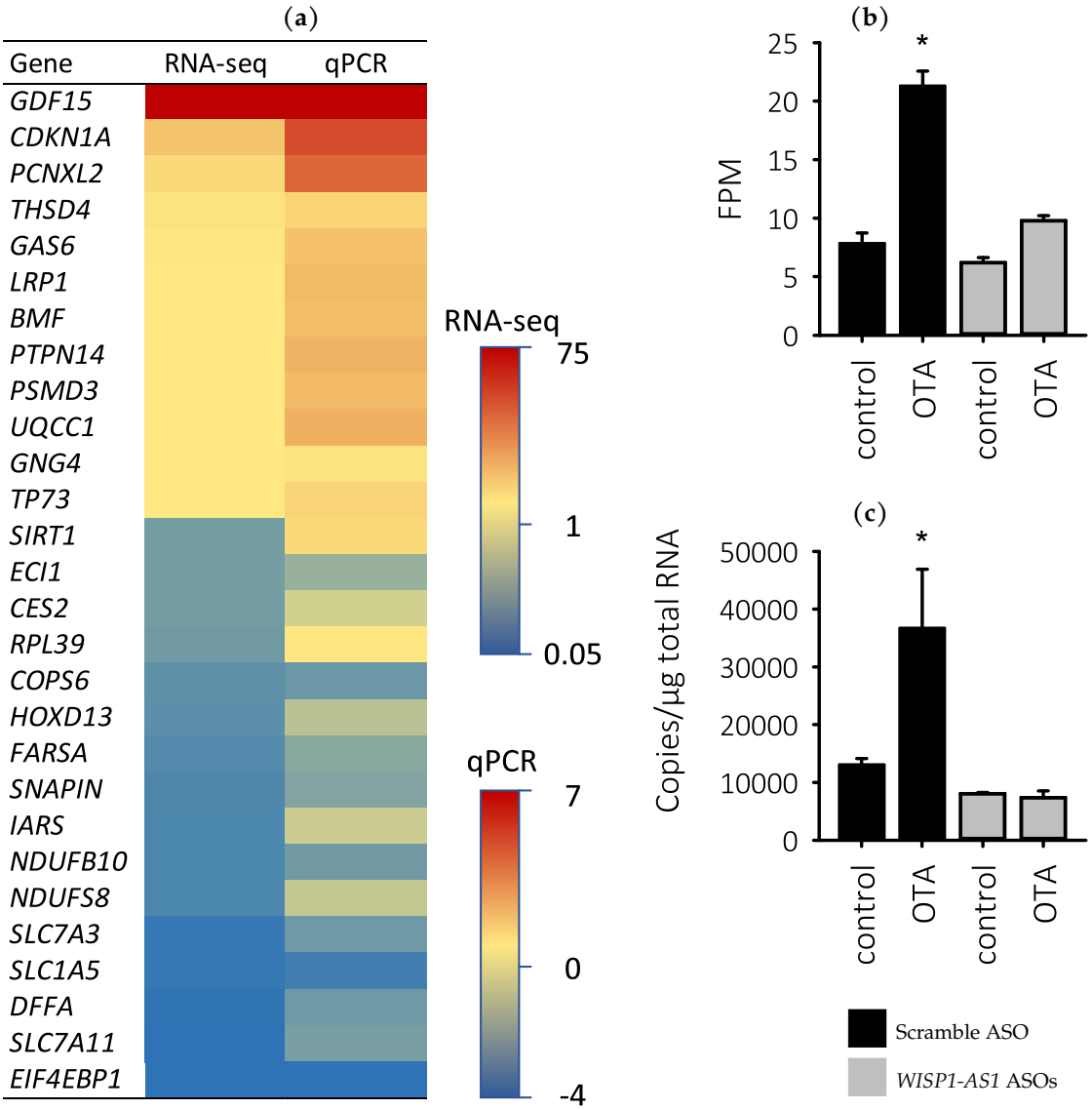


Figure 4.11: Validation of some genes from RNA-seq. (a) Some genes from RNA-seq were validated by qPCR. In 93 % of cases, genes showed the same pattern of expression (up- or down-regulation). From red to blue, color indicates decline in expression. RNA-seq data are expressed as FC in the OTA/scramble ASO group, in comparison with the control/scramble ASO group. Values below one represent downregulation. qPCR data is presented as ΔΔCt in the OTA/scramble ASO group, in comparison with the control/scramble ASO group. Values below zero indicate downregulation. n = 4 (RNA-seq), 2-7 (qPCR). (b) Growth-arrest specific gene 6 (*GAS6*) mRNA was upregulated in a *WISP1-AS1*-dependent manner in RNA-seq and (c) confirmed by ddPCR. Data are shown as means ± SEM. Asterisk indicates significant difference ($P < .05$) from the control/scramble ASO group. n = 4 (RNA-seq), n = 2 (ddPCR).

creased activation as downstream caspase-3 in cells with decreased *WISP1-AS1* expression (the OTA/*WISP1-AS1* ASOs group) (Fig. 4.12b). OTA-induced, *WISP1-AS1*-dependent effect on caspase-9, a mediator of mitochondrial apoptotic pathway, was opposed to caspases 2 and 3. The activity of caspase-9 was

significantly lower in the presence of *WISP1-AS1* (in the OTA/scramble ASO group) in comparison with the control/scramble ASO group (Fig. 4.12d). The data indicate a protective role of *WISP1-AS1* against mitochondrial pathway of apoptosis.

Other caspases showed either very low activity (caspase-8, Fig. 4.12c), no significant changes (caspase-10, Fig. 4.12e) or a pattern clearly different from caspase-3 (caspase-12, Fig. 4.12f).

All caspase activities were normalized to total cellular protein content. Cells treated with OTA resulted in a global protein reduction, independently of *WISP1-AS1* (Fig. 4.12g). OTA reduced total protein content down to 72 % and 78 % in the control/OTA and the ASO/OTA group, respectively, in comparison with the corresponding controls. A slight reduction in total cellular protein was previously shown by SAUVANT ET AL. 2005A with 100 nM OTA on opossum kidney cells.

4.5.2 *WISP1-AS1* influence on nuclear fragmentation

In apoptosis, characteristic changes in cell morphology include chromosomal DNA fragmentation and, in the late phase, nuclear fragmentation. The latter can be visualized by microscopy. Therefore, to evaluate nuclear fragmentation in OTA-induced, *WISP1-AS1*-dependent fashion, nuclei were stained with DAPI and analyzed by Cytation™ 3 Cell Imaging Multi-Mode Reader to quantify small nuclear fragments.

The control/scramble ASO group have shown very low percentage of small nuclear fragments, only 2.9 ± 2.2 % out of total number of nuclei in the observation field. In OTA-treated cells with *WISP1-AS1* presence (the OTA/scramble ASO group), nuclear fragmentation stayed low, with basically the same percentage of small nuclear fragments. In contrast, in OTA-treated cells with decreased *WISP1-AS1* expression (the OTA/*WISP1-AS1* ASOs group), nuclear fragmentation was significantly increased to 10.4 ± 3.2 % (Fig. 4.12h).

The results are consistent with and support the increased caspase-3 activity in OTA-treated cells with decreased *WISP1-AS1* expression (the OTA/*WISP1-AS1* ASOs group) (Fig. 4.12a). Moreover, these results demonstrate that in the presence of *WISP1-AS1*, cells were "protected" against apoptosis. Accordingly, I suggest *WISP1-AS1* function as an antiapoptotic lncRNA.

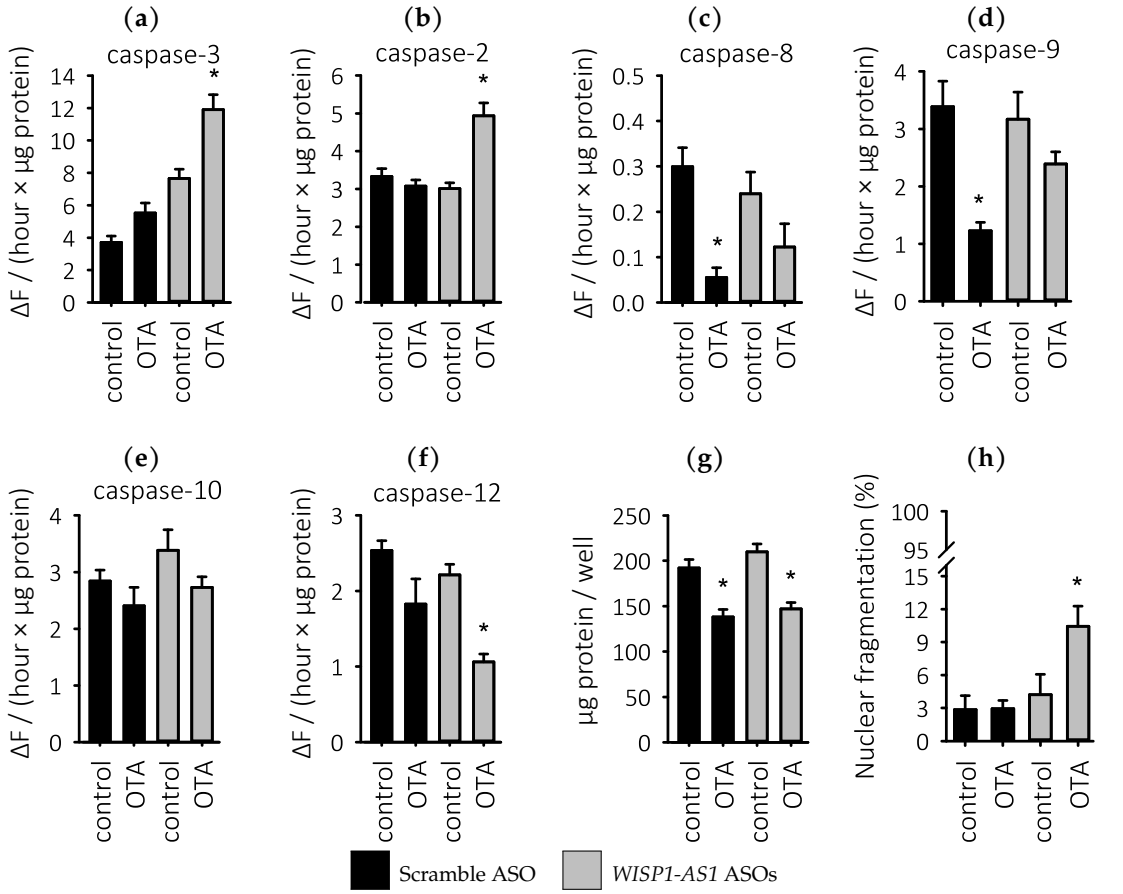


Figure 4.12: Changes in apoptotic events: caspase activities and nuclear fragmentation in the cells treated with OTA and ASOs. Caspase activities were measured by using florigenic substrates. In OTA-treated cells with *WISP1-AS1* knockdown, **(a)** caspase-3 activity was increased three times in and **(b)** caspase-2 activity was significantly increased in comparison with the control/scramble ASO group. **(c)** The activity of caspase-8 was decreased in the *WISP1-AS1* presence (in the OTA/scramble ASO group) but a whole caspase-8 expression was at a low scale. **(d)** Caspase-9 was decreased in OTA-treated cells in the presence of *WISP1-AS1*. **(e)** The activity of caspase-10 did not show significant difference between four groups. **(f)** Caspase-12 activity decreased in OTA-treated cells with *WISP1-AS1* knockdown. **(g)** OTA decreases total protein in the cell, independently of *WISP1-AS1*. The amount of protein per well was used to normalize caspase activities. **(h)** Nuclear fragmentation was obtained by Cytation™ 3 Cell Imaging Multi-Mode Reader after staining the nuclei with DAPI. Nuclear fragmentation shows increased percent of small nuclear fragments in the OTA-treated cells in the absence of *WISP1-AS1*. Data in all graphs are shown as means ± SEM. Asterisk in all graphs indicates significant difference ($P < .05$) from the control/scramble ASO group. n = 12–16 in all graphs.

4.5.3 Necrotic cell death

An alternative pathway to apoptotic cell death is necrosis. Upon necrotic cell damage, cell membranes become leaky. The release of lactate dehydrogenase (LDH), an intracellular enzyme, serves as a measure of leaky membranes and, therefore, a measure of necrosis.

In OTA-treated cells in the presence of *WISP1-AS1* (the OTA/scramble ASO group), cells show slight but significant increase in necrosis (Fig. 4.13a). When the expression of *WISP1-AS1* was decreased in OTA-treated cells (the OTA/*WISP1-AS1* ASOs group), cells did not show prominent necrosis.

Taken together, these findings indicate that necrosis only occurred in OTA-treated cells when *WISP1-AS1* was present, in other words, when the cells were prevented from going into apoptosis, possibly due to the potential *WISP1-AS1* antiapoptotic effect. In the case of prevented apoptosis, cells might shift to necrosis.

Therefore, slight increase in LDH release might represent a compensatory cell death pathway in OTA-treated cells with *WISP1-AS1* expression.

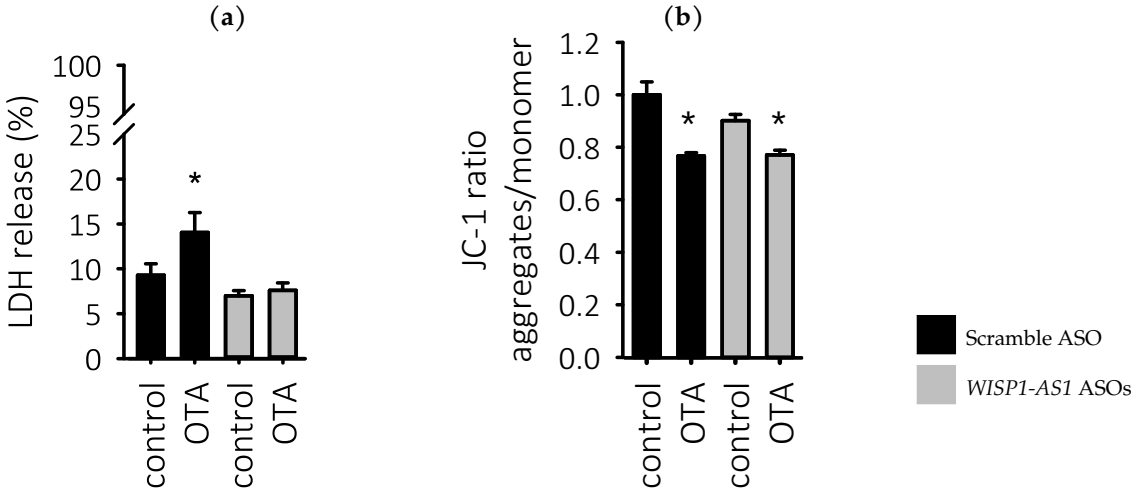


Figure 4.13: *WISP1-AS1* influence on the cellular necrotic death and mitochondrial polarization. **(a)** The percentage of LDH release from the cells is expressed as the activity of LDH in the cell medium relative to a sum of LDH activity in cell lysate and cell medium. Slight increase in LDH release was observed in the OTA-treated cells in the presence of *WISP1-AS1* (the OTA/scramble ASO group), in comparison with the control/scramble ASO group. Slight increase in LDH release might be a compensatory cell death pathway in OTA-treated cells with *WISP1-AS1* expression where apoptosis is reduced. n = 18. **(b)** JC-1 dye is a mitochondrial membrane potential-sensitive. JC-1 ratio of aggregates/monomers indicates decrease in the mitochondrial membrane potential in OTA-treated cells, independently of the presence of *WISP1-AS1*. Data in both graphs are shown as means ± SEM. Asterisk in both graphs indicates significant difference ($P < .05$) from the respective control. n = 4 in both graphs.

4.5.4 Changes in mitochondrial membrane potential

To investigate whether *WISP1-AS1* is a mediator of OTA influence on the mitochondria, I assessed mitochondrial membrane potential by using the membrane potential-sensitive JC-1 dye and Cytation™ 3 Cell Imaging Multi-Mode Reader.

Control cells, treated with scramble ASO or *WISP1-AS1* ASOs but without OTA, showed intense red fluorescence from JC-1 aggregates, an indication of healthy mitochondria with a largely negative mitochondrial membrane potential. However, cells treated with OTA, independently of *WISP1-AS1*, displayed stronger green fluorescence from JC-1 monomers and less red fluorescence, an indication of less negative mitochondrial membrane potential.

The ratio of red/green fluorescence (corresponding to aggregates/monomer ratio) show the reduction of mitochondrial membrane potential by 20 % in the cells treated with OTA, independently of *WISP1-AS1* presence (Fig. 4.13b). The action of OTA on mitochondrial membrane potential is not mediated by *WISP1-AS1*; it is solely OTA effect.

4.6 Cellular glucose consumption and lactic acid formation

4.6.1 *WISP1-AS1* influence on energy metabolism

Cellular glucose demands can change in the growing cells but also in pathological processes. Glucose metabolism can directly influence signaling pathways of cell death, in particular apoptosis. In the studies of apoptosis in Sections 4.5.1 and 4.5.2, antiapoptotic effect of *WISP1-AS1* was observed.

Accordingly, the next step was to investigate presumed influence of OTA-induced *WISP1-AS1* on the energy metabolism. Therefore, I measured glucose consumption to seek for changes in energy demands between OTA-treated cells containing *WISP1-AS1* and cells lacking *WISP1-AS1*.

Glucose consumption of the control/scramble ASO group during 3 h in HEPES-Ringer buffer was $18.3 \pm 2.4\,\mu$mol/mg of protein (Fig. 4.14a). Cells treated with OTA in the presence of *WISP1-AS1* consumed an almost double amount of glucose in comparison with the control/scramble ASO group ($33.4 \pm 5.1\,\mu$mol/mg of protein). When *WISP1-AS1* was knocked down by ASOs in OTA-treated cells (the OTA/*WISP1-AS1* ASOs group), glucose consumption dropped to the level of control cells.

These observations imply that *WISP1-AS1* is involved in high glucose consumption.

4.6.2 Lactate production and glutaminolysis

In different (patho)physiological processes, cells can switch their glucose metabolism from oxidizing pyruvate (a product of glucose breakdown) in

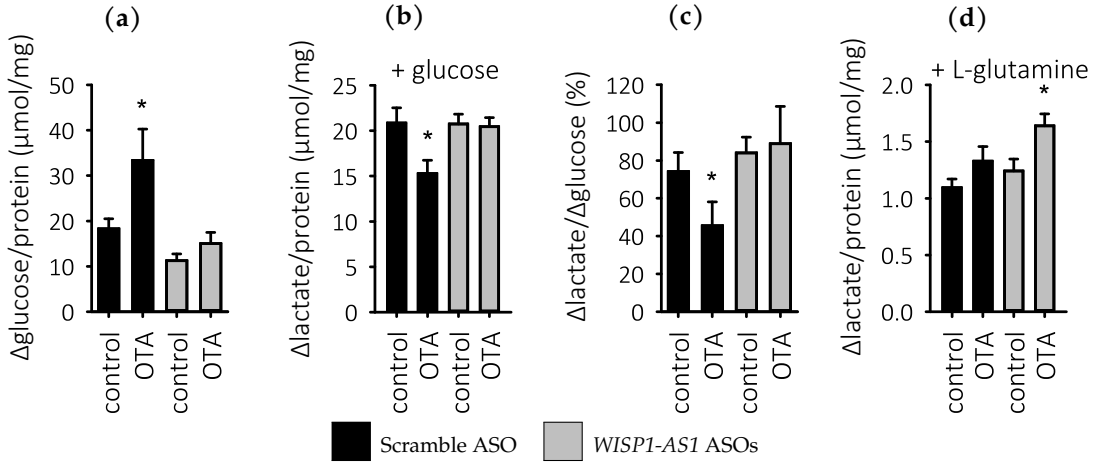


Figure 4.14: *WISP1-AS1* influence on glucose consumption and lactate production. **(a)** To measure glucose consumption, cells were incubated in HEPES-Ringer buffer with 11 mM glucose for 3 h. OTA-treated cells in the presence of *WISP1-AS1* consume significantly more glucose, but **(b)** they produce lower amount of lactate from it. **(c)** Low lactate production results in a low lactate per glucose ratio in the OTA/scramble group. **(d)** Lactate can be produced from other compounds than glucose, for example, glutamine. Cells incubated with 11 mM L-glutamine in a HEPES-Ringer buffer (without glucose) for 3 h show an increase in lactate production in OTA-treated cells with *WISP1-AS1* knockdown. Data in all graphs are shown as means ± SEM. Asterisk in all graphs indicates significant difference ($P < .05$) from the scramble ASO/control group. n = 12 in all graphs.

the tricarboxylic acid (TCA) cycle to aerobic glycolysis. The latter produces excessive lactate, also in the presence of oxygen, which can be measured in the cell culture media.

OTA-treated cells in the presence of *WISP1-AS1*, when compared with the control/scramble ASO group, consumed high amounts of glucose (Fig. 4.14a) and produced remarkably low amount of lactate from glucose during 3 h in HEPES-Ringer buffer (Fig. 4.14b). Based on this, lactate per glucose ratio was significantly lower in the OTA/scramble ASO group, in comparison with the control/scramble ASO group (Fig. 4.14c).

In contrast to the group that contains *WISP1-AS1*, OTA-treated cells with *WISP1-AS1* knockdown produced a high amount of lactate (20.4 ± 1.0 μmol/mg of protein; Fig. 4.14b) from a relatively low amount of consumed glucose (15.0 ± 5.3 μmol/mg of protein; Fig. 4.14a). This amount of lactate is more than it can possibly be synthetized from glucose.

Lactate can originate from other compounds than pyruvate synthesized from glucose. The major compound (other than pyruvate) that contributes to the lactate production is glutamine. Cells can consume glutamine, resulting in a slightly higher lactate production in the cells treated with OTA, especially with-

out *WISP1-AS1* (Fig. 4.14d). Glutaminolysis can partially explain why cells have more lactate than can be produced from glucose.

In conclusion, it appears that OTA-treated cells with *WISP1-AS1* expression employ the TCA cycle to obtain energy from glucose. This conclusion is based on an observation that pyruvate (from glucose) is not completely converted to lactate. Therefore, pyruvate is probably oxidized into CO_2 via the TCA cycle. Without *WISP1-AS1*, OTA-treated cells show increased aerobic glycolysis as a form of obtaining energy. Increased aerobic glycolysis is often observed in cancer cells.

5 Discussion

About 20 years ago, it was still believed that the majority of human genome belongs to a "junk DNA", DNA that does not have structural or transcriptional function. Nowadays, clearly most of this "junk" turned out to be regulatory elements or genes for ncRNAs. Knowledge gained within last 20 years about noncoding transcripts accomplished a separation of housekeeping and regulatory ncRNAs into several categories. The category of lncRNAs represents abundant and diverse transcripts longer than 200 nt. LncRNAs are mainly predicted by high throughput tools, reaching almost a number of 150 000 in the human genome. Alas, only to a small number of those predicted lncRNAs a function was ascribed.

In this work I described a novel lncRNA, *WISP1-AS1*, and attributed it several possible functions as the outcome of the experimental work.

5.1 *WISP1-AS1* molecular characteristics

Some *WISP1-AS1* characteristics have been previously described [Dittmar 2017; Hennemeier 2014] but prior to my project, *WISP1-AS1* orientation was unknown. The reason for it is the discovery by qPCR experiment which cannot distinguish between the orientation of the amplified DNA fragment. Generally speaking, lncRNAs can be oriented in sense or antisense in respect to the closest protein-coding gene. To distinguish between the orientation in sense or antisense of the newly discovered transcript, strand-specific reverse transcription (RT) followed by qPCR is a straightforward method. By strand-specific primers in RT reaction, I was able to identify *WISP1-AS1* orientation in the opposite direction in comparison with *WISP1* mRNA. This lists *WISP1-AS1* among the natural antisense transcripts (NATs), well-known and common functional transcripts with essential functions in human health. The examples are *AIRN* and *NEAT1*, essential for maintaining monoallelic gene expression and nuclear paraspeckle formation, respectively [reviewed by Latos et al. 2012;Lo et al. 2016]. Other NATs are increased expressed after cellular exposure to cell stressors including mycotoxins and thus participate in etiology of human diseases through various mechanisms [Wanowska et al. 2018].

Due to all included controls, I am confident that the results are reliable. First, the results of the RT with an antisense primer specific for the sense-oriented transcript did not detect any signal. Second, genomic DNA control confirmed that strand-specific primers are functional. Together, the results have demonstrated that sense primer in RT detected the only possible transcript, and this is *WISP1-*

AS1 in antisense. Control reactions without RTase and without primers confirmed technically good experiments. The discovery of *WISP1-AS1* transcription in antisense was the crucial finding of my doctoral dissertation; all the subsequent experiments and findings depended on it.

WISP1-AS1 expression in OTA-treated cells was confirmed by Northern blot. Likewise, Northern blot confirms its length and orientation in antisense in comparison with *WISP1* mRNA. Northern blot performed with a negative control probe, a probe with a sequence identical to *WISP1-AS1*, did not show any signal. This is due to the lack of base pair compatibility between a negative probe and *WISP1-AS1*. Hybridization controls indicated orientation-specificity of probes and confirmed well-produced probes and reliability of results.

Long noncoding RNAs are often RNA Pol II transcripts, therefore they contain a poly(A) tail and thus are similar to mRNAs. LncRNAs without poly(A) tail also exist and are usually transcribed by RNA Pol III. Nonpolyadenylated RNAs are less identified and investigated simply because many experiments to identify novel lncRNAs were performed on the poly(A) fraction of RNA, mostly to avoid sequencing of abundant nonpolyadenylated rRNAs. In the case of *WISP1-AS1*, analyzing two different types of RNA, total RNA and polyadenylated RNA, isolated from OTA-treated cells identified polyadenylation of *WISP1-AS1*. This is a confirmation that *WISP1-AS1* has a poly(A) tail. To support the reliability of oligo$(dT)_{18}$ primer priming in the poly(A) tail, and not inside the molecule I analyzed the sequence of *WISP1-AS1*. There are no internal stretches longer than seven adenosine bases in a row. Therefore, I conclude that qPCR results are reliable since oligo$(dT)_{18}$ primer should not bind to such a short stretch of adenosines.

To investigate if it is possible to confirm that *WISP1-AS1* can be expressed in antisense in comparison with mRNA, I searched an online ENCODE Matrix database. Evidence of *WISP1-AS1* expression in antisense was found in NHEK cells. These data were available from the RNA-seq experiments performed on NHEK cells and uploaded into the database. Such evidence shows that antisense transcription from the *WISP1* locus exists and that it is possible in the nontreated cells. In other words, it is naturally occurring. The reason that *WISP1-AS1* is not shown in other cell lines might be because of the specific OTA treatment. As I explained in introduction (Section 1.7.3) and showed throughout results, *WISP1-AS1* expression is elevated only in epithelial renal cells in OTA toxicity and tumor transformation. This such a narrow window might present an obstacle for the appearance of *WISP1-AS1* in RNA-seq experiments that are usually (vastly) found in online databases. I am not aware of the RNA-seq results on

OTA-treated human renal epithelial cells other than ours. At least, I did not find experiments with nanomolar amounts of OTA analyzing a whole transcriptome. There are, however, some experiments on human renal cells treated with OTA, but they were looking for miRNA changes by RNA-seq. One experiment, performed by Limonciel et al., used targeted RNA-seq called TempO-seq on RPTEC cells treated with various amounts of OTA [2018]. By checking their online available TempO-seq data, I found that *WISP1* gene was not detected.

When two transcripts overlap in opposite directions, like antisense *WISP1-AS1* overlaps the 3′ end of the *WISP1* mRNA, then one transcript can be functionally blocked. These two transcripts are transcribed *in cis* and their sequence complementarity is 100 %, without mismatches. This kind of complementarity is hard to achieve with a transcript acting on another transcript *in trans*. Nontreated HEK293T cells were analyzed to observe the expression pattern of these two transcripts. Interestingly, in control cells, both transcripts were nonexpressed. After OTA induction, only *WISP1-AS1* was increased expressed and *WISP1* mRNA was not detectable. This differential expression of *WISP1-AS1* confirms that OTA is a *WISP1-AS1*-specific inducer. However, this experiment cannot show potential *WISP1-AS1* suppression of *WISP1* mRNA. Conditions had to be created when both transcripts were induced. For that, OTA and PMA were used and they specifically induce *WISP1-AS1* and *WISP1* mRNA, respectively. If, in *WISP1-AS1* presence, mRNA expression suddenly strongly decreases, that would be a confirmation that *WISP1-AS1* can suppress mRNA when both transcripts are induced. Since that was not the case, these two transcripts, transcribed convergently from the *WISP1* locus, do not influence each other's expression. This calls attention to different cellular functions of *WISP1-AS1*.

Since *WISP1-AS1* transcribes in antisense from the 3′ end of the *WISP1* gene, there exists a possibility that *WISP1-AS1* is an extension from the upstream gene, *NDRG1*. If *WISP1-AS1* would be a transcriptional extension from the *NDRG1* locus, then their expression pattern would be similar. However, *WISP1-AS1* transcription seems to be entirely inside the *WISP1* locus. In conclusion, *WISP1-AS1* and *NDRG1* have independent transcription. This confirms *WISP1-AS1* existence as a separate transcript, in particular, antisense lncRNA in *WISP1* locus.

To support noncoding potential of *WISP1-AS1*, I analyzed an online database on translational events in the *WISP1* locus. There was no translation outside exons, and exon translation corresponds to *WISP1* mRNA. Thus, the chance that *WISP1-AS1* translates is minimal. Ribosome profiling analyzes nascent protein chain, and it does so independently of the orientation of the RNA molecule rela-

tive to its gene. Therefore, since no translation events were visible in intron 4 nor in 3′ UTR from where *WISP1-AS1* transcribes, I took those results as a support of noncoding function of *WISP1-AS1*. From the analyzed database, GWIPS-viz and previous Western blot experiments performed with different antibodies by HENNEMEIER ET AL. 2012, there is a proof of *WISP1-AS1* noncoding characteristic strong enough to believe *WISP1-AS1* is noncoding.

5.2 *WISP1-AS1* expression in renal cancer cells with and without OTA induction

Among the chemicals tested so far, only OTA achieved strong upregulation of *WISP1-AS1*. Other stress inducers (forskolin, hydrogen peroxide, ionomycin, SIN1 and SNAP) did not have a significant effect on *WISP1-AS1* expression [unpublished data, personal communication with Dr. Gerald Schwerdt]. On the other hand, OTA is known carcinogenic agent. For that reason, I investigated *WISP1-AS1* expression in renal *cancer* cells, ccRCC. High *WISP1-AS1* expression was detected in renal *cancer* cells without OTA induction. This finding points out that *WISP1-AS1* might play a role in carcinogenesis independently of OTA. For some lncRNAs, mechanisms of their involvement in carcinogenesis are described. An example is ZFPM2-AS1[1], upregulated in gastric cancer. ZFPM2-AS1 inhibits nuclear translocation of p53, thus acts antiapoptotic and has a direct role in carcinogenesis [KONG ET AL. 2018]. Several publications revealed MAPK pathway being constitutively active in different RCC cell lines and tumor biopsies [HUANG ET AL. 2008; OKA ET AL. 1995; RAO ET AL. 2019]. This could be the way to transcriptionally activate *WISP1-AS1* in cancer cells independently of OTA, but such a suggestion remains to be investigated. In ccRCC cells, despite *WISP1-AS1* expression independent of OTA, OTA is able to further induce expression of *WISP1-AS1*. It can be only speculated that OTA-induced carcinogenic transformation of renal cells depends on *WISP1-AS1* upregulation.

To study the clinical relevance of upregulated *WISP1-AS1* in renal cancer cells, it would be worth to analyze urine, blood and biopsies of human renal tumor patients as well as patients with OTA-suspected nephropathies for *WISP1-AS1* expression. From the collected data, it could be evaluated whether *WISP1-AS1* could serve as a prognostic or diagnostic biomarker or therapeutic target in treatment of ccRCC and OTA-induced nephropathies.

Some lncRNAs are already biomarkers or targets for therapeutic approaches in human diseases [reviewed by BARMAN ET AL. 2019; FATEMI ET AL. 2014; LEUCCI

[1] zinc finger protein, FOG family member 2-antisense transcript 1

2018; Van der Hauwaert et al. 2019; Wanowska et al. 2018].

5.3 Potential *WISP1-AS1* functions

WISP1-AS1 knockdown was established with LNA™ GapmeR antisense oligonucleotides (ASOs), a third generation of ASOs used for successful lncRNA knockdown. This approach is suitable for analysis of changes in gene expression after a target molecule is knocked down. It is widely used as tool in recent years and some ASOs hold promising as therapeutics for various human diseases [Shimojo et al. 2019]. In the conditions of reduced *WISP1-AS1* expression down to 10 % it was possible to observe cellular changes dependent on *WISP1-AS1*. Knockdown by the ASO tool was used in all subsequent experiments to investigate *WISP1-AS1* functions.

5.3.1 *WISP1-AS1* influence on transcriptome and TFs

WISP1-AS1 influence on the transcriptome is indicated by many genes differentially expressed (DE) in a *WISP1-AS1*-dependent manner. This is in accordance with many other NATs, whose primary function is regulation of gene expression by various mechanisms. Because *WISP1-AS1* causes changes in gene expression, it signifies its implication in pretranscriptional, transcriptional or posttranscriptional gene regulation. Since *WISP1-AS1* localizes in the nucleus, where all such gene regulation processes occur, it is possible that *WISP1-AS1* acts on the availability of epigenetic modifiers, TFs and/or RNA Pol II. In the end, it is no wonder that nuclear lncRNAs are called "key regulators of gene expression" [Q. Sun et al. 2018] and it seems like *WISP1-AS1* is one of them.

Genes that appeared DE in RNA-seq were analyzed for enrichment in gene ontology (GO) terms. The purpose was to find gene clustering which would aid in explaining *WISP1-AS1* function. Unfortunately, there was no clusters enriched within GO categories. Such result points out that *WISP1-AS1* is not affecting components of a certain pathway or cellular process. More likely, it causes changes in only one or few transcripts through which it exerts a biological function.

The aim to find common elements in DE genes was successful in case of the analysis of promoter regions for the overrepresentation of TFBS. Certain TF can regulate a gene if the gene promoter contains response element (i.e. TFBS) for this TF. Two different *in silico* analysis, by g:profiler and *pscan*, recognized significantly overrepresented TFBSs for E2F, EGR-1 and SP1 in more than 70 % of

WISP1-AS1-dependent up- and downregulated genes. Additionally, both analysis identified ELK-1 in more than 70 % of downregulated genes. This means that genes responsive to *WISP1-AS1* are regulated by those TFs. Further, it suggests that *WISP1-AS1* might function as a modulator of TF activity. All identified TFBSs, overrepresented in up- and downregulated genes, were GC-rich. This is consistent with GC-rich promoter regions around TSS [YAMASHITA ET AL. 2005] which can get methylated on CpG sites. Additionally, this might explain *WISP1-AS1* influence on preferentially those TFs. However, more investigations are needed to understand this process.

To confirm that *WISP1-AS1* can affect differential expression of genes by modulating the action of TFs, promoter activity assays were performed. Results showed that the activity of E2F and EGR-1 was affected by OTA in a *WISP1-AS1*-dependent manner. E2F is a family of transcription activators and repressors [J. D. JOHNSON AND G. 2006]. EGR-1 is a transcription activator, promoting survival and suppressing apoptosis when is overexpressed, seen in cancers [VIROLLE ET AL. 2003]. If *WISP1-AS1* promotes EGR-1 and on the other hand it suppresses E2F transcription repressors, then *WISP1-AS1* promotes protection against apoptosis and cell survival.

To confirm reliability of the promoter assays, another TF was chosen, AP-1, that was not significantly overrepresented in *WISP1-AS1*-dependent DE genes. AP-1 activity was not affected by OTA, and therefore not by OTA-induced *WISP1-AS1*. Thus, AP-1 confirms that results are reliable and TFBS found in *in silico* analysis are not showing by chance in *WISP1-AS1*-dependent DE genes. SP1 is reported to activate genes through interactions with other DNA-bound TFs, and one of them is E2F [reviewed by SAFE AND ABDELRAHIM 2005]. So, it seems like SP1 and E2F work together to activate gene transcription. This might be the reason why both appeared enriched in *WISP1-AS1*-dependent DE genes. Another research describes the simultaneous activation of SP1, E2F and ETF. All three TFs are involved in proliferation and dependent on the active MAPK pathway [ZELLMER ET AL. 2010]. In conclusion, identified TFs must be an important way of *WISP1-AS1* action on gene expression.

The implication of lncRNAs in TF regulation is a suggestion that cells have advantages from employing an additional regulatory process. Cells do not have to use all the machinery to produce "expensive" proteins but instead transcribe lncRNAs. LncRNAs can act on TFs to regulate gene expression and induce fast cellular response. Maybe this is one of the reasons why lncRNAs are cell- and tissue-specific and why lncRNAs are as relevant as proteins in gene expression regulation [MARCHESE AND RAIMONDI 2017].

RNA-seq revealed *WISP1-AS1* influence on TF activity. However, it did not result in gene clustering. Because of that, it was unknown whether other cellular functions are affected by *WISP1-AS1*. Different functional assays had to be employed to obtain an insight into *WISP1-AS1* consequences on cell metabolism and survival. Experiments were conducted in the presence of OTA, with or without ASOs to knockdown *WISP1-AS1* and with appropriate controls. This experimental setup allows to observe changes of the cellular functions in the presence and absence of *WISP1-AS1*, discussed below.

5.3.2 *WISP1-AS1* influence on cell survival

When *WISP1-AS1* was present in OTA-treated cells, caspase-3 activity was low. This is an indication that cells maintain low rate of apoptosis. To confirm that low caspase-3 activity results in reduced apoptosis, Cytation 3 Cell Imaging Multi-Mode Reader was used that combines digital microscopy and conventional microplate detection. With this system, low nuclear fragmentation was obtained representing reduced apoptosis and supporting caspase-3 results. In OTA-treated cells with *WISP1-AS1* knockdown, apoptosis was elevated be means of increased both caspase-3 activity and nuclear fragmentation. In conclusion, all results indicate that *WISP1-AS1* has a protective role against apoptosis. Low caspase-3 activity in the presence of *WISP1-AS1* resulted in a slightly elevated necrosis as a compensatory pathway.

Different initiator caspases were analyzed to reveal which one is involved in the apoptotic pathway in the *WISP1-AS1* absence. From five tested, only caspase-2 showed the same pattern as caspase-3. Caspase-2 has a unique property to localize to the nucleus. It belongs to the nuclear apoptotic pathway which can be induced for example by DNA damage. Besides a role in apoptosis, caspase-2 was reported to have various nonapoptotic functions in cells [FAVA ET AL. 2012; KUMAR 2009]. It would have to be investigated in more detail what is the connection between *WISP1-AS1* and caspase-2. Still, it is tempting to speculate that *WISP1-AS1* action in the nucleus maintains gene expression and prevents apoptosis, which in turn keeps low levels of activated caspase-2. Knockdown of *WISP1-AS1* in the nucleus is probably triggering caspase-2 activation. How exactly is not known yet. Possibly the lack of *WISP1-AS1* protective role on nuclear processes lead to activation of caspase-2 pathway. Caspase-2 may perform functions in cell cycle arrest and DNA damage-induced apoptosis.

Along with an antiapoptotic act by preventing caspase-3, *WISP1-AS1* showed protective role against mitochondrial apoptotic pathway. In earlier study of

OTA action on human renal cells [SCHWERDT, FREUDINGER, SCHUSTER, ET AL. 2003], it was noticed that OTA "spares" mitochondria and does not lead to disruption of respiratory chain nor cytochrome c release. These data could be fulfilled with *WISP1-AS1* influence on reducing caspase-9 activity and thus preventing apoptosis and the accompanying mitochondrial changes. This could be OTA-induced, *WISP1-AS1*-dependent protective role on mitochondria. However, cytochrome c release and influence on other components of mitochondrial apoptotic pathway should be investigated in cells with and without *WISP1-AS1* knockdown.

GAS6 gene, validated after RNA-seq, might mediate *WISP1-AS1*-dependent antiapoptotic effect observed in OTA-treated cells. GAS6 can lower caspase-3 activity and promote cell survival [HASANBASIC ET AL. 2004]. *GAS6* is positively regulated in *WISP1-AS1*-dependent manner. In the OTA-treated cells in the presence of *WISP1-AS1*, *GAS6* mRNA is upregulated. In the absence of WISP1-AS1, *GAS6* mRNA is downregulated. This negatively correlates with the caspase-3 activity: in the absence of *WISP1-AS1*, when *GAS6* is downregulated, caspase-3 activity is higher. Accordingly, I propose a mechanism where *WISP1-AS1* downregulates apoptosis by inducing upregulation of *GAS6*.

Many lncRNAs showed to positively regulate cancer cell survival by downregulating apoptotic processes [reviewed by DE PAEPE ET AL. 2018]. There are examples of well-known lncRNAs that act oncogenic (*MALAT1*, *H19* and *ANRIL*[2]). *ZFPM2-AS1* promotes proliferation and suppresses apoptosis of gastric cancer cells *in vitro* [KONG ET AL. 2018]. It remains to be investigated whether *WISP1-AS1* functions as an oncogenic NAT.

5.3.3 *WISP1-AS1* influence on glucose metabolism

OTA-treated cells in the *WISP1-AS1* presence consume significantly more glucose than control cells. These results might indicate a *WISP1-AS1* role in regulating glucose metabolism in the cells. Glucose transporters (GLUTs) facilitate transport of glucose into the cell. Contrary to the expectations, it was not possible to support the changes observed in glucose consumption with RNA-seq results. In RNA-seq, mRNAs for *GLUT* were not found significantly upregulated in *WISP1-AS1*-dependent manner. Therefore, upregulated transcription of *GLUT* mRNA is not an underlying mechanism responsible for higher glucose consumption in OTA-treated cells in the *WISP1-AS1* presence. This is in contrast to cancer cells which often upregulate *GLUT* mRNA [reviewed by SZABLEWSKI

[2] antisense non-coding RNA in the ihibitors of cyclin-dependent kinase 4 (INK4) locus

2013]. Additionally, some lncRNAs are shown to be able to upregulate *GLUT* trascription, for example *ANRIL* [Zou et al. 2016].

OTA-treated cells consume a lot of glucose and produce lower amount of lactate, possibly due to proper glucose oxidation. In those cells, *WISP1-AS1* might shift cellular energy metabolism towards the active TCA cycle but this is only a speculation. Anyhow, this would mean that cells with *WISP1-AS1* maintain the TCA cycle and OXPHOS to obtain maximum amount of energy in the form of ATP. In conclusion, it seems like OTA-treated cells shift glucose metabolism towards mitochondria and this is dependent on *WISP1-AS1*.

On the other hand, from the results it seems like in OTA-treated cells with *WISP1-AS1* knockdown, cellular energy production changes. Judging from the excess lactate production, cells probably switch to aerobic glycolysis, also known as the Warburg effect [reviewed by Devic 2016; Vander Heiden et al. 2009]. This would mean that in the absence of *WISP1-AS1*, cells cannot shift their energy metabolism towards mitochondria and instead utilize aerobic glycolysis. Physiologically, cells obtain aerobic glycolysis for fast growth and rapid ATP production during proliferation.

Cells with *WISP1-AS1* knockdown produce high amounts of lactate, even more than it is possible to produce from the amount of consumed glucose. Results show that other compounds than glucose can be consumed by those cells and contribute to lactate production. Without *WISP1-AS1*, cells consume significantly higher amount of glutamine than control cells. One of the fates of glutamine in the cells is glutaminolysis to form lactate. Higher glutamine consumption is observed in cancer cells [Hosios et al. 2016], indicating *WISP1-AS1* might play a role in maintaining energy metabolism of a healthy cell.

Cancer cells tend to generate energy through aerobic glycolysis and they have several benefits from it. They use a part of glucose for conversion into ATP, but the rest is used to produce precursors of macromolecules as well as coenzymes, for example NADPH which protects from oxygen radicals [Vander Heiden et al. 2009]. Additionally, maintaining glycolysis helps tumor cells in formation of an acidic micromilleau for growth, invasion and metastasis. Avoiding OXPHOS prevents accumulation of oxigen radicals [reviewed by Hua et al. 2018; Yu et al. 2015]. Metabolic reprogramming to glycolysis is therefore one of the hallmarks of cancer [Hanahan and Weinberg 2011].

Some lncRNAs are shown to regulate metabolic reprogramming by deciding whether glucose is directed into glycolysis or the TCA cycle. Examples of lncRNAs exist that support cellular switch to glycolysis [reviewed by Hua et al. 2018; Yu et al. 2015]. Some tumors, however, keep obtaining ATP via functional

mitochondria capable of OXPHOS or can use in parallel OXPHOS and aerobic glycolysis [reviewed by DE PAEPE ET AL. 2018]. *SAMMSON*[3] is a lncRNA that maintains tumor cells obtaining energy via OXPHOS. It does so by directly influencing p32 responsible for maintaining mitochondrial membrane potential and OXPHOS [LEUCCI ET AL. 2016]. *WISP1-AS1* might have an influence on the active TCA cycle by some similar mechanism to *SAMMSON*. Then *WISP1-AS1* would have an oncogenic role but more research is needed to clarify this role.

5.3.4 *WISP1-AS1* influence on mitochondrial membrane potential

Mitochondrial membrane potential in the OTA-treated cells was slightly reduced, independently of *WISP1-AS1*. To measure it, membrane potential-sensitive dye JC-1 was used. JC-1 is a fluorescent dye that serves to semiquantitatively measure relative changes of mitochondrial membrane potential in living cells. Other dyes, for example, rhodamine-based and $DiOC_6$ are less specific [experimentally tested by MATHUR ET AL. 2000] and depend on other factors, like the size of the cell [reviewed by LY ET AL. 2003]. Rhodamine-123 is a qualitative dye that distributes passively between the cytosol and mitochondria, whereas JC-1 changes color with membrane potential. Consequently, JC-1 posses ratiometric fluorescence, offered by no other mitochondrial membrane potential indicator [KEIL ET AL. 2011]. Therefore, signal-to-noise ratio with JC-1 dye is much better than other systems to obtain relative changes in mitochondrial membrane potential.

From the studies of OTA effects on different human epithelial kidney cells (e.g. IHKE cells) [SCHWERDT, FREUDINGER, SCHUSTER, ET AL. 2003], it is known that OTA mildly affects mitochondrial membrane potential. Mild uncoupling of mitochondria could serve to protect excessive production of reactive oxygen species [reviewed by ZOROV ET AL. 2014]. *WISP1-AS1* is not involved in OTA-induced change in mitochondrial membrane potential but it possibly keeps OTA-treated cells to still utilize OXPHOS. Only without *WISP1-AS1*, cells have deregulated glucose demands and switch their metabolism away from mitochondria.

LncRNAs are existing that directly influence mitochondria and its functions, like membrane potential, mitochondrial apoptosis and production of reactive oxygen species [reviewed by DE PAEPE ET AL. 2018], but it seems like *WISP1-AS1* is not one of them. OTA influence on mitochondria is independent of *WISP1-AS1* and has no detrimental outcome on mitochondria (mitochondrial mem-

[3] survival associated mitochondrial melanoma specific oncogenic noncoding RNA

brane potential is slightly depolarized but there is no mitochondrial apoptosis and seems like oxidative respiration are not affected).

5.4 Proposed *WISP1-AS1* mechanism

OTA enters the cell through OAT and activates ERK, a part of MAPK pathway, responsible for OTA-induced signaling pathway. Activated ERK is suspected to induce *WISP1-AS1* transcription in antisense in comparison to the *WISP1* gene [HENNEMEIER ET AL. 2012]. *WISP1-AS1* have several functions in the cell. One function is influence on the TFs. OTA-induced, WISP1-AS1-dependent DE genes contain binding sites for TFs EGR-1 and E2F.

As shown by the promoter activity assays, *WISP1-AS1* upregulates the activity of EGR-1. EGR-1 is a transcription activator known to influence genes responsible for proliferation, cell survival and prevention of apoptosis [VIROLLE ET AL. 2003]. By activating EGR-1, *WISP1-AS1* could have a role in cell survival. Another TF confirmed by promoter activity assay is E2F, and this is a family of transcription activators and repressors [J. D. JOHNSON AND G. 2006]. *WISP1-AS1* downregulated the activity of E2F in the promoter activity assays. One possibility is that *WISP1-AS1* represses the activity of E2F6, a transcription repressor.

Next, OTA-treated cells with *WISP1-AS1* showed a reduced apoptosis. *GAS6* might be a functional link of *WISP1-AS1* antiapoptotic action. *GAS6* is positively regulated in *WISP1-AS1*-dependent manner. In the promoter region of *GAS6* mRNA, a binding site for E2F6 exists. *WISP1-AS1* might reduce E2F6 activity and thus prevent it from binding to the promoter in *GAS6* gene. This would activate *GAS6* transcription. Translated GAS6 protein would exert its function on caspase-3 and reduce its activity [HASANBASIC ET AL. 2004]. The resulting effect is *WISP1-AS1*-dependent *GAS6* reduction in apoptosis.

Besides acting antiapoptotic by upregulating GAS6 mRNA through action on TFs, *WISP1-AS1* might have a direct, so far unknown, mechanism to directly reduce activity of caspase-2 and caspase-9. By preventing caspase-9 activity, *WISP1-AS1* might prevent mitochondrial apoptosis.

In OTA-treated cells with *WISP1-AS1* upregulation, higher glucose consumption was recorded. However, GLUT mRNAs were not upregulated in RNA-seq data. OTA-treated cells do not produce much lactate, so I propose that *WISP1-AS1* directs cellular energy metabolism towards mitochondria, towards the TCA cycle and OXPHOS. This way, OTA-treated cells with *WISP1-AS1* presence would maintain normal energy metabolism.

In OTA-treated cells, with *WISP1-AS1* knockdown, cells produce excessive lactate, which is probably exported out of the cell by monocarboxylate transporter 4 (MCT4) [Contreras-Baeza et al. 2019]. From this observation, I conclude that cells with *WISP1-AS1* knockdown cannot shift energy metabolism towards mitochondria. Instead, they utilize energy through an aerobic glycolysis, often seen in tumors. The observation brings me to the conclusion that *WISP1-AS1* regulates cellular energy metabolism and in its presence, cells can maintain normal energy metabolism.

The above described mechanisms are schematically presented in Fig. 5.1.

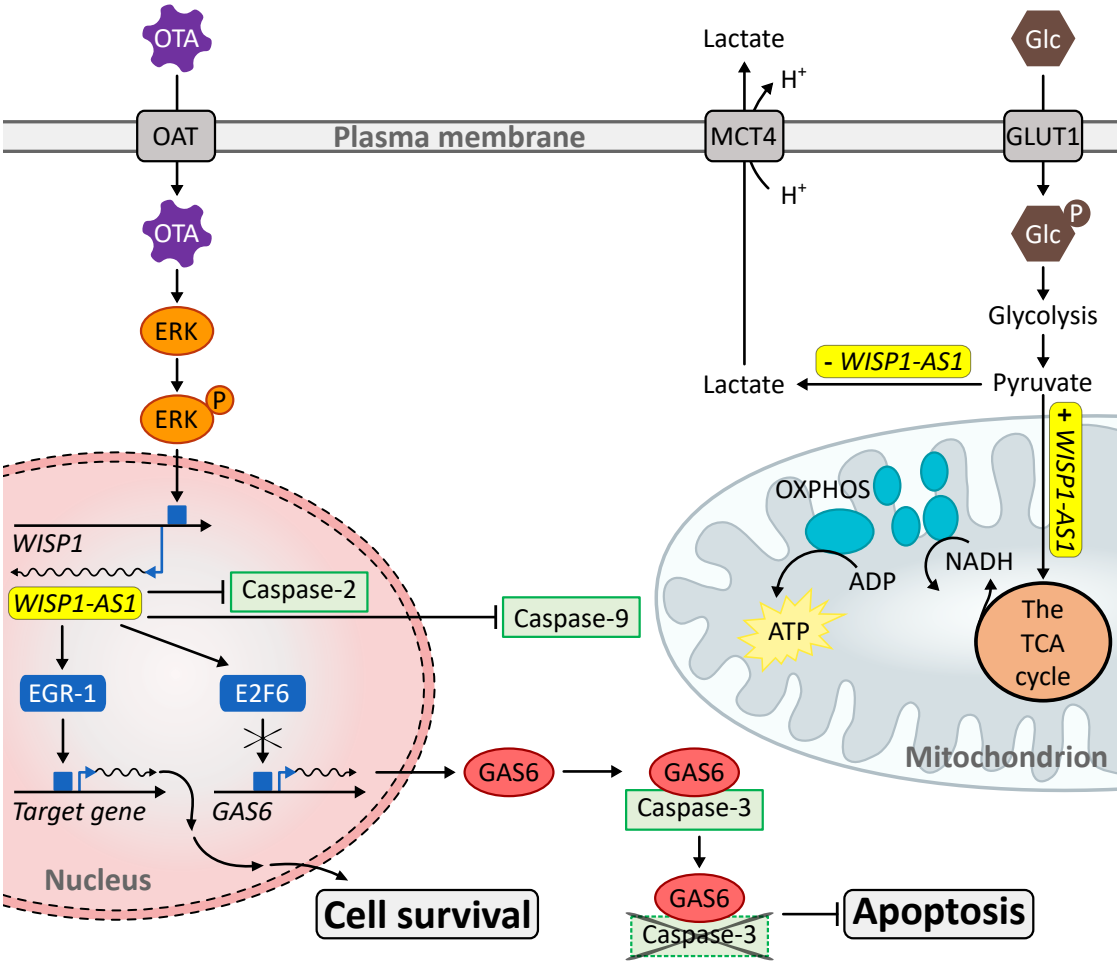


Figure 5.1: A speculative model of *WISP1-AS1* functions in OTA-treated cells. Functions are described above in the text

The future relies on the understanding of *WISP1-AS1* mechanisms and how its function affects cellular changes in OTA-treated cells. The question remains to be answered: can we prevent detrimental effect of OTA on human health by finding *WISP1-AS1* functions?

Appendix A Sequences

Table A.1: Sequences of antisense oligonucleotides

Type	Sequence (5′ to 3′)	Length, nt
Against *WISP1-AS1*	TCGTTAGCTGAGTGAG	16
	CAGTGGAAGGTAGCGT	16
	ACTTGCTTGGATGAAG	16
	TGTGAAGCTGAAAGTA	16
	TCTTAACTGAAAACGT	16
Against "sense" *WISP1-AS1*	CCGCAAGTGTGAAGTT	16
	GAGCATTCCACCTTAC	16
	ACTTGAGATTTACTGT	16
	AGATCTAGGCTGTATG	16
	TTGGACTTGCATTACG	16
Scramble ASO	AACACGTCTATACGC	15

Table A.2: Sequences of Northern blot probes

Type	Sequence (5′ to 3′)	Length, nt
Probe against *WISP1-AS1*	AGGCAGGAGGGGCGGTTAGGGCAAAGCGTGTCCCCTGAGTGTGG AGTCCCGATGCCAGTTCTGCCACTAGCTAGCCAGGGGAACTTGC TTGGATGAAGGAGCTACATAGGCTCCCCATGTTGGTGAAACCAT GATGAATGACTGGCCATAGGCAAGACAAAAATGCAGGACAAAAG AGAAACTGCAGGCTGTGAGATACAACCCAAACAAAACATTTTTC TCTGTGGTCCTCCATTTACCCCTGTGCGGTATGCTGGATCTCAC TGTGTTTGCTGTTGTCCCTCCTTGCTTGAGGATGCTGTGAAGCT GAAAGTAAGGTGGAATGCTCCCACATAGTGAGAAGGGAAAAACT GGGGGCTCAGGG	364
Negative control probe	CCCTGAGCCCCCAGTTTTTCCCTTCTCACTATGTGGGAGCATTC CACCTTACTTTCAGCTTCACAGCATCCTCAAGCAAGGAGGGACA ACAGCAAACACAGTGAGATCCAGCATACCGCACAGGGGTAAATG GAGGACCACAGAGAAAAATGTTTTGTTTGGGTTGTATCTCACAG CCTGCAGTTTCTCTTTTGTCCTGCATTTTTGTCTTGCCTATGGC CAGTCATTCATCATGGTTTCACCAACATGGGGAGCCTATGTAGC TCCTTCATCCAAGCAAGTTCCCCTGGCTAGCTAGTGGCAGAACT GGCATCGGGACTCCACACTCAGGGGACACGCTTTGCCCTAACCG CCCCTCCTGCCT	364
Probe against *ACTB*	Biotin-CTCATTGTAGAAGGTGTGGTGCCA	24

Table A.3: Sequences of primers

Purpose	Gene name	Sense primer (5′ to 3′)	Antisense primer (5′ to 3′)	Product length, bp
Northern blot probe	*WISP1* intron 4 (I)[c]	AGGCAGGAGGGGCGGTTAGG	CCCTGAGCCCCCAGTTTTTCC	364
Antisense-orientation-specific RT	*WISP1* intron 4 (II)	GTGTCCCCTGAGTGTGGAGT	–	–
Sense-orientation-specific RT[a]	*WISP1* 3′ UTR	–	CACACCCCTACCTGAACCTG	–
Polyadenylation RT	Oligo$(dT)_{18}$ primer	–	TTTTTTTTTTTTTTTTT	–
PCR after strand-specific RT	*WISP1* intron 4 (III)	CCCTCCTTGCTTGAGGATGCTG	CCCTGAGCCCCCAGTTTTTCC	85
	WISP1 exon 5[d]	ACTACACCCAAGCCTGATCC	TGACCTTTCCCTGTCTCTGG	498
Total RNA + oligo$(dT)_{18}$/RP[b]	*WISP1* intron 4 (IV)	AAGGTACCAGCTGCAGAGGA	CTGTATGGCGGCATGAAGCTTG	177
Poly(A) RNA + oligo$(dT)_{18}$/RP	*WISP1* intron 4 (V)	GTGTCCCCTGAGTGTGGAGT	CCCTGAGCCCCCAGTTTTTCC	337
WISP1 mRNA	*WISP1* exon 1 – exon 2	AGAGGTGGTCGGATCCTCTG	CCAGTGGAGCTGGGGTAAAG	176
Upstream of *WISP1-AS1*	*NDRG1*[e]	CTGCACCTGTTCATCAATGC	AGAGAAGTGACGCTGGAACC	341
Reference genes	*18S*	CTCAACACGGGAAACCTCAC	CGGACATCTAAGGGCATCAC	268
	GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG	107
	ACTB	GCACAGAGCCTCGCCTT	CCTTGCACATGCCGGAG	112
TaqMan primers	*WISP1-AS1* (intron 4)[f]	GCTGTGAGATACAACCCAAA	CAAGCAAGGAGGGACAAC	105
TaqMan probe	Probe (FAM-labeled)	ACACAGTGAGATCCAGCATACCGCA	–	–
TaqMan primers	*WISP1* exon 1 - exon 2[f]	GGCATGAGGTGGTTCCTG	CTGGGGTAAAGTCCATGGTC	112
TaqMan probe	Probe (HEX-labeled)	AGCAGCAGCCGCCAGCACCG	–	–
RNA-Seq validated genes	*BMF*	GAGGTACAGATTGCCCGAAA	CGATTTTGGTTCTGCTGGTG	86
	CDKN1A	ACTGTCTTGTACCCTTGTGC	CTCTTGGAGAAGATCAGCCG	144
	CES2	ATTTGCCAAGCCACCTCTAG	TAGATGCTGAGGTACAGGCA	201
	COPS6	GAGTGACTGGGAGTGTTTCC	AACTCCAGCTCCTTGAACAC	262
	DFFA	AGTTTGTGGCATTGGCTAGT	TAGGAGGATGATGCTGGACA	173
	ECI1	AAAGACACCCTGGAGAACAC	TGGAGATGAAGCTGACGAAG	280
	EIF4EBP1	GGGAGGTACCAGGATCATCT	TGTTTTGGTCACAGGTGAGT	70
	FARSA	CATGGAGGTGTTCAGCTACC	TCCCGGATATTGTTGATGCC	180

Primers (continued)

Purpose	Gene name	Sense primer (5′ to 3′)	Antisense primer (5′ to 3′)	Product length, bp
RNA-Seq validated genes (continued)	*GAS6*	AAAGTGAACACGAGGATGCA	ACGACTTCTACTTCCCAGGT	143
	GDF15	CTCCAGATTCCGAGAGTTGC	CACTTCTGGCGTGAGTATCC	130
	GNG4	CACCACTAGCATCTCCCAAG	GGCACTGGAATGATGAGAGG	150
	HOXD13	TCGACATGGTGTCCACTTTC	ATGTCCGGCTGATTTAGAGC	190
	IARS	CTCCTTACTTCGGTGCTGAG	GCAAACAGGGAGTGAGTCTT	77
	LRP1	CATGGACTTCAGCTATGCCA	AGTAGAAGTTGCCTGTCAGC	191
	NDUFB10	ATGATGAAAGCGTTCGACCT	TTGCACTCAGTGATGTCTGG	137
	NDUFS8	GAACCGGCCACCATCAACTA	TGTCGATGTCATAGCGGGTG	208
	PCNXL2	GACGAGTGGGTATTTGCTGA	CTGCTGGGTCTTCATACTCG	121
	PSMD3	ATCAAGGAGCACGTGAAACA	GGAAGGGGAGCAAAAAGTCT	181
	PTPN14	AAGAGCCTGTGAAGGAGAGA	GGTGGCATCAACTCGATTCT	203
	RPL39	AGGATTAAGCGATTCCTGGC	CCAGCTTGGTTCTTCTCCAA	127
	SIRT1	TGACACTGTGGCAGATTGTT	ACATGAAACAGACACCCCAG	152
	SLC1A5	CTGAAGGGGAGCTGCTTATC	TCCTCCATCTCCACGATCTT	133
	SLC7A3	TGTGCTATGCGGAGTTTGGT	TGGACCCCTGCAGAGTCTTA	210
	SLC7A11	GGGGTCCTGTCACTATTTGG	CCCAGACTCGTACAAAAGC	128
	SNAPIN	GACGCGTTGTCTTGGTTAAC	GAATCCAGCATTGCTCTCCT	109
	THSD4	TGACTACTTAGGCTCCGACA	AATTGCCCAGTTCCCATTGA	214
	TP73	TTGAGGTCACTTTCCAGCAG	GACACCTTGATCTGGATGGG	118
	UQCC1	GGAAGTATCACACCACACGT	TTCAAAGGTCCCGTGAATCC	118

[a] Reverse transcription

[b] Random primer (hexamer)

[c] $T_{ann} = 64.0\,^{\circ}C$

[d] $T_{ann} = 63.5\,^{\circ}C$

[e] F3/R1 primer pair taken from Ghalayini et al. 2013.

[f] $T_{ann} = 59.0\,^{\circ}C$

Table A.4: *WISP1-AS1* sequence. 5′ to 3′ orientation. Black part is representing 3′ UTR; red part is representing exon 5; dark gray part is representing intron 4 part of the *WISP1* gene.

AGGGAAAAATGACATTAAAAAATCAAGCCATTTATTGCAATGTTTTCCTATTCTGACACATTTGGAAGCTCTAAAAACA
GGGGGAAAATATGGGTTATAAAGACAGTCCAATTTCGATCTGAATCCTAACTTTCCCAGTCACCAACTGGGTGACTCTG
GTTGAGTTTTCTCACCTCTATGAGCTTCCGTTTCACATTTGTAAGAACAGAAATAATAATGGGAACTTCTCAACATTGT
CGTGAAGATTCAGTGAGCTAATACATGCAAAGCACTGAGAGCAATGTCTGGCACAGAAAAGGCCCTAAATAAAAGAATT
CTAACAGTAAATATTATATTTTCCTAATACAATAAATAATAGCCTGATATTCATTAACAACAACCTTTTCCTTTTAGCT
TCAACCTCTTCAGCTTTAAACCTTTATTAAGTCTATTTCTGTTTCTAAACAACCGGTAAACCTCCATCTTCCTACCAGA
AAACTGGCCATTTGGTCAGCAGAACGAAAGGCTGTGAAATGAGTCTCTGCAATAGATGTGTCCTGAACACGGGGGCCCC
TGGAGTCCCACAGCCTCCAGCTCTAGAGGTCACCCCAGGCTGCTACCAAGGGCACCCACTTCTTGGTTTGACCCACACC
CCTACCTGAACCTGTCTTGGTCATTTCAGTGGGTCTGGCATGGTCTGCAGCTGGGTCTCTCAAGGCTCTGTAAGAGTAG
AGGAGTGTTCCAGGGCACCACTCTCAGGGGCCAGACAGGCCAGTCCAGAGGCCTTACCAATTCCTATGAACCCCAAGCA
GGACAAGGGAGAAGATTCAAGGAAGAGCCTGGCCAACTGCCCAAAGCCCTGCCAGTCAGCAGCTGATTCAGTGTGAGAG
TCAATTGGTCCTTCTGAAACGGGCTGACCTTTCCCTGTCTCTGGAGTATTAACTGGGGGCTTTTCCCATCAACAGGAAT
GTTTATCAAATTTTATTTCTGACTGGGACAAACCTAATCTGTACCCTTCACAGTGTAGGGGTGAAGAGAAACTTAACCT
CAACAATGCCCAGGTTAATGGATACAACTTTCCATTAAAAATCAAAATTAAGCAACTATTCAACTCACTTGGCAAATGT
TTGATGACCTTGAGGTGCATACATATTTGGAATCAGGATAGTTTTGGTGCGCTGACGTCAGAATTACCCATTACAGTAC
CTCTTGGCAGGCTCCAAATAATAGATTATTCTATTGTTCCAAATTCCATCTATTCATCTGAATGGACCCAAAGAGTCTT
GTGGTTTGGCATCTAAAGAAGAAATTAGTGATATTAAAAGTCTACCTGATTCCATTTCTTGGGATTTAGGCAAGATCCA
GCAGGACTTCTAGTGACTTGGAAAGGCTGGATCAGGCTTGGGTGTAGTCCAGAACAGCTAGAGACAGGCATTTTTCTTT
AGAGTAGATGCTGAATGATATCAAGGAGAAAACTCATAGCATGGGCCTGAGCAGCACCATCAGGCGTCATTTGAATGGT
TAGAGACAGAAATGGAGGCCAAGGGTCCAGATCAGGGTAACTAAGGCTCATTGGTGAAAAGTTATTGGCCATAAGGGCT
GACTGCTTCACAGGCATTGGGTTAGTCCCCAAGACCCAAGATTTGTGCCTGCCTAGTTGGCAATTTCTGAGAAGTCAGG
GTAGGATTCCAAGTCAGCAAAGATGTCATTGGGATTCCTACAGCTCAGGTTACAGAAGCAGGCATTAATCCATAGGACC
TGGCGGGAGAAGCCAAGCCCATCAGGACACTGGAAGGACACGTCGATAGTCTTAGACTTGTAGGGGATGCAGCACCTAT
TGTCCATGCAAACTCCACAGTACTTGGGTTGATAGGAGCGTGTGCTGATGCAGCCCGCAAGTGTGAAGTTCATGGATGC
CTCTGGCTGGTACACAGCCAGACACTTCTTCCCTGCCTGAAGGAAAGGAAAGGAGCTTTCAGTGGGAGTGCTCAGAGAA
TGGACAACCACCTTCTTCCCCTGAGCCCCCAGTTTTTCCCTTCTCACTATGTGGGAGCATTCCACCTTACTTTCAGCTT
CACAGCATCCTCAAGCAAGGAGGGACAACAGCAAACACAGTGAGATCCAGCATACCGCACAGGGGTAAATGGAGGACCA
CAGAGAAAAATGTTTTGTTTGGGTTGTATCTCACAGCCTGCAGTTTCTCTTTTGTCCTGCATTTTTGTCTTGCCTATGG
CCAGTCATTCATCATGGTTTCACCAACATGGGGAGCCTATGTAGCTCCTTCATCCAAGCAAGTTCCCCTGGCTAGCTAG
TGGCAGAACTGGCATCGGGACTCCACACTCAGGGGACACGCTTTGCCCTAACCGCCCCTCCTGCCTTTGCCTCTCGTGG
CCTTTCACACATACCTTTAGTATTCAACGTATTCAAATGGTTGACATTAAAGGTAACGCTACCTTCCACTGGGCTATGG
GCCACTTGAGATTTACTGTTCTTTCTGTGTTTCTGTGGTATAATTCCATGCCTAGTACAGAGTAGGCACTTACTAAACC
CATTGGATTGAGGGTTCTCAGGGTCTACTGTGTAAATAGTATGCTAGATCTAGGCTGTATGGCGGCATGAAGCTTGCCC
AATTTGGGAAACCCTCTGAGGGCACATAATTCTGGAAAAAAATTGGTTGGAAATCAAAATTGGCTTGGACTTGCATTAC
GTGGTCCAGTAATGGTAGAGCCATCTCACTCAGCTAACGACCTCTGCTGTCATTCCTCTGCAGCTGGTACCTTTTCCAT
GTACGTTTTCAGTTAAGAGATGTCTATGGAATGCTTACTGTATACCAAGCGCAGAAAATACAAAAGCTAGTATTACAGG
GAAAAATAATCA

Appendix B Acronyms

18S	small ribosomal unit part; S is Svedberg unit
2D	two-dimensional
$\mathbf{2^{\Delta\Delta Ct}}$	fold change in gene expression
3′	3 prime
5′	5 prime
786-O	clear cell renal cell carcinoma cell line 786-O
A-to-I	adenosine-to-inosine
A230	absorbance at 230 nm
A260	absorbance at 260 nm
A280	absorbance at 280 nm
ACTB	beta actin
ADAMTSL-6	A disintegrin and metalloproteinase with thrombospondin motifs-like protein 6
ADAR	adenosine deaminase acting on RNA
AFC	7-amino-4-trifluoromethyl coumarin
AGO2	Argonaute 2
AIRN	antisense to insulin-like growth factor type 2 receptor RNA noncoding
Alu	from lat. *Arthrobacter luteus*
ANOVA	Analysis of Variance
ANRASSF1	antisense to Ras association domain family member 1
ANRIL	antisense non-coding RNA in the ihibitors of cyclin-dependent kinase 4 (INK4) locus
AP-1	activator protein 1
ASO	antisense oligonucleotide
Asp	aspartate
ATCC	American Type Culture Collection
ATP	adenosine-5′-triphosphate
BACE1	beta-site amyloid precursor protein cleaving enzyme 1
BACE1-AS	beta-site amyloid precursor protein cleaving enzyme 1-antisense transcript
BCA	bicinchoninic acid
BEN	Balkan endemic nephropathy
BHQ-1	Black Hole Quencher 1
BLAST	basic local alignment search tool
BMF	B-cell lymphoma 2 (BCL2) modifying factor
bp	base pair

BSA	bovine serum albumine
c-MYC	cellular myelocytomatosis oncogene
Ca^{2+}	calcium ion
$CaCl_2 \cdot {}_2H_2O$	calcium chloride dihydrate
caspase	cysteine-dependent, aspartate-specific peptidase
CCN	cellular communication network factor
ccRCC	clear cell renal cell carcinoma
CDKN1A	cyclin dependent kinase inhibitor 1a
cDNA	complementary DNA
°C	degree Celsius
CES2	carboxylesterase 2
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CO_2	carbon dioxide
COPS6	constitutive photomorphogenesis 9 (COP9) signalosome subunit 6
CpG	cytosine-phosphate-guanosine
CREB	cAMP-response element binding protein
Ct	cycle threshold
CTP	cytidine-5′-triphosphate
Cu^{+}	cuprous ion
Cu^{2+}	cupric ion
DAG	diacylglycerol
DAPI	4,6-diamidino-2-phenylindole
ΔCt	delta cycle threshold
ΔΔCt	delta delta cycle threshold
ddPCR	droplet digital polymerase chain reaction
DE	differential expression
ΔF	change in fluorescence
DEPC	diethyl pyrocarbonate
DFFA	DNA fragmentation factor subunit alpha
DINO	DNA-damage-induced noncoding lncRNA
$DiOC_6$	3,3′-dihexyloxacarbocyanine iodide
DIRAS3	guanosine-5′-triphosphate-binding protein Di-Ras3
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DNMT	DNA methyltransferase
dNTP	deoxyribonucleotide triphosphate

DPPA	developmental pluripotency-associated protein 2 gene
dsRNA	double-stranded RNA
DTT	dithiothreitol
DUM	developmental pluripotency-associated 2-upstream binding muscle lncRNA
e.g.	*lat. exempli gratia*; for example
E2F	transcription factor necessary for the transactivation of the adenoviral E2 promoter
ECI1	enoyl-coenzyme A delta isomerase 1
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGR-1	early growth response protein 1
ELK-1	ETS like-1 protein
EMT	epithelial-mesenchymal transition
ENCODE	encyclopedia of DNA elements
endo-siRNA	endogenous short interfering RNA
ERK	extracellular signal-regulated kinase
et al.	*lat. et allii*; and others
ETF	embryonic TEA domain-containing factor
FAM	6-fluorescein amidite
FARSA	phenylalanyl-tRNA synthetase subunit alpha
FC	fold change
FCS	fetal calf serum
FDR	false discovery rate
FENDRR	Forkhead box protein F1 adjacent non-coding developmental regulatory RNA
FGFR3	fibroblast growth factor receptor 3
FGFR3-AS1	fibroblast growth factor receptor 3-antisense transcript 1
FPM	fragments per million
FRAM	free right Alu monomer repeat element
g	gram
GADD45A	growth arrest and DNA-damage-inducible 45 alpha
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAS6	growth-arrest specific gene 6
GC	guanosine-cytosine
GDF15	growth differentiation factor 15
gDNA	genomic DNA
GLUT	glucose transporter
GNG12-AS1	G protein subunit gamma 12-antisense RNA 1

GNG4	G protein subunit gamma 4
GO	gene ontology
GOrilla	gene ontology enrichment analysis and visualization tool
GTP	guanosine-5′-triphosphate
GWIPS-viz	genome-wide information on protein synthesis visualized
h	hour
$\mathbf{H^+}$	proton
H19	*H19* long noncoding RNA
½-sbsRNA	half Staufen 1-binding site RNA
HEK293T	human embryonic kidney 293T
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEX	hexachlorofluorescein
HindIII	*Haemophilus influenzae Rd* III
HOTAIR	homeobox transcript antisense RNA
HOTTIP	homeobox A transcript at the distal tip
HOX	homeobox
HOXA	homeobox A gene cluster
HOXC	homeobox C gene cluster
HOXD	homeobox D gene cluster
HOXD13	homeobox protein D13
HTT	huntingtin
HTT-AS	huntingtin-antisense transcript
i.e.	*lat. id est*; that is
IARS	isoleucyl-tRNA synthetase 1
ICE	imprinting control element
IGF2R	insulin-like growth factor type 2 receptor
IGV	Integrative Genomics Viewer
IHKE	immortalized human kidney epithelial
IkB	inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells
JC-1	5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-benzimidazolcarbocyanine iodide
JNK	c-jun N-terminal kinase
J/cm^2	Joule per square cm
kb	kilobase
KCl	potassium chloride
L	liter
LDH	lactate dehydrogenase
lincRNA	long intergenic noncoding RNA

LNA	locked nucleic acid
lncRNA	long noncoding RNA
LRP1	low density lipoprotein receptor-related protein 1
M	molar
mA	milliampere
MALAT1	metastasis associated lung adenocarcinoma transcript 1
MAPK	mitogen activated protein kinase
MCT4	monocarboxylate transporter 4
MDCK-C7	Madin-Darby canine kidney cellular subtype (clone) 7
mg	milligram
$\mathbf{MgCl_2}$	magnesium chloride
$\mathbf{MgCl_2 \cdot {}_6H_2O}$	magnesium chloride hexahydrate
MGFP	monster green fluorescent protein
µg	microgram
µg/µl	microgram per microliter
µg/ml	microgram per milliliter
µl	microliter
µm	micrometer
µM	micromolar
min	minutes
miR-485-5p	micro RNA 485, forward strand
miRNA	micro RNA
ml	milliliter
mm	millimeter
mM	millimolar
MOPS	morpholinoethanesulfonic acid
MPN	mycotoxic porcine nephropathy
mRNA	messenger RNA
n	number of samples per group
$\mathbf{Na_2HPO_4 \cdot {}_2H_2O}$	disodium hydrogen phosphate dihydrate
NaCl	sodium chloride
$\mathbf{NAD^+}$	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
NAPDH	nicotinamide adenine dinucleotide phosphate, reduced
$\mathbf{NaH_2PO_4 \cdot {}_2H_2O}$	sodium dihydrogen phosphate dihydrate
$\mathbf{NaHCO_3}$	sodium bicarbonate
NAT	natural antisense transcript
NAT6531	natural antisense transcript 6531
NCBI	The National Center for Biotechnology Information

ncRNA	noncoding RNA
NDRG1	N-Myc downstream regulated gene 1
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 10
NDUFS8	NADH:Ubiquinone Oxidoreductase Core Subunit S8
NEAT1	nuclear paraspeckle assembly transcript 1
NEB	New England Biolabs
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NF-YA	nuclear transcription factor Y subunit alpha
ng	nanogram
NheI	*Neisseria mucosa heidelbergensis* I
NHEK	normal human epidermal keratynocyte
NHLF	normal human lung fibroblasts
NKILA	nuclear factor kappa-light-chain-enhancer of activated B cells interacting long noncoding RNA
nm	nanometer
nM	nanomolar
nt	nucleotide
OAT	organic anion transporter
oligo(dT)$_{18}$	18-mer oligonucleotide deoxythymine
ORF	open reading frame
OTA	ochratoxin A
OTA^{-}	monoanionic ochratoxin A
OTA^{2-}	dianionic ochratoxin A
OXPHOS	oxidative phosphorylation
P	probability value
p38	p38 mitogen-activated protein kinase
p53	tumor protein 53
PAI-1	plasminogen activator inhibitor type 1
PANDAR	p21 associated ncRNA DNA damage activated RNA
PBS	phosphate-buffered saline
PCA3	prostate cancer antigen 3
PCNXL2	pecanex-like protein 2
PCR	polymerase chain reaction
PDHX	pyruvate dehydrogenase complex component X
%	percent
pH	lat. *potentia hydrogenii*; measure of acidity
Phe	phenylalanine
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)

PIWI	P-element induced wimpy testis
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
poly(A)	polyadenylated
PPP1R12A	phosphatase 1 regulatory subunit 12A
PRC2	polycomb repressive complex 2
pRNA	promoter-associated RNA
PRUNE2	prune homolog 2 with BCH domain
PS	phosphatidylserine
PSMD3	proteasome 26S subunit, non-ATPase 3
PTPN14	protein tyrosine phosphatase non-receptor type 14
PU.1	PU.1 transcription factor
pUC18	plasmid University of California 18
qPCR	quantitative polymerase chain reaction
R12A-AS1	antisense to phosphatase 1 regulatory subunit 12A
RACE	rapid amplification of cDNA ends
RASSF1A	Ras association domain family member 1
RCC	renal cell carcinoma
rDNA	ribosomal DNA
RFP	red fluorescent protein
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RNAi	RNA interference
RNA Pol II	RNA polymerase II
RNA Pol III	RNA polymerase III
RNase	ribonuclease
RPL39	ribosomal protein L39
RPMI	Roswell Park Memorial Institute
RPTEC	renal proximal tubule epithelial cells
rRNA	ribosomal RNA
RT	reverse transcription
RTase	reverse transcriptase
s	second
SAMMSON	survival associated mitochondrial melanoma specific oncogenic noncoding RNA

SAPK	stress-activated protein kinase
SBS	Staufen 1 binding site
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SERPINE1	serpin family E member 1
SIN1	3-morpholinosydnonimine hydrochloride
Sineb2	short interspersed elements B2
SINEUP	antisense RNA that require short interspersed elements B2 to upregulate translation
siRNA	short interfering RNA
SIRT1	sirtuin 1
SLC1A5	solute carrier family 1 (neutral amino acid transporter), member 5
SLC711	solute carrier family 7 (anionic amino acid transporter light chain, Xc-system), member 11
SLC7A3	solute carrier family 7 (cationic amino acid transporter, Y+-system), member 3
SMD	Staufen homolog 1-mediated mRNA decay
SNAP	S-nitroso-N-acetylpenicillamine
SNAPIN	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) associated protein
SP1	specificity protein 1
ssRNA	single-stranded RNA
STAU1	Staufen homolog 1
SYBR	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
T7	bacteriophage T7
TaqMan	*Thermus aquaticus* (*Taq*) Polymerase + PacMan
TARID	transcription factor 21 antisense RNA-inducing demethylation
TBE	Tris-borate-EDTA
TCA	tricarboxylic acid
TCF21	transcription factor 21
TF	transcription factor
TFBS	transcription factor binding site
THSD4	thrombospondin type 1 domain containing 4
×	times

× g	times Earth's gravitational force; unit of relative centrifugal force
TNF	tumor necrosis factor
TP73	tumor protein P73
TPA	12-O-tetradecanoyl-phorbol-13-acetate; see PMA
Tris	tris(hydroxymethyl)aminomethane
Triton-X-100	2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol
TSS	transcription start site
Uchl1	ubiquitin carboxyl-terminal hydrolase isozyme L1
UDG	uracil-DNA glycosylase
U/µl	unit per microliter
UQCC1	ubiquinol-cytochrome C reductase complex assembly factor 1
UTP	uridine-5'-triphosphate
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
WISP1	WNT1-inducible signaling pathway protein 1
WISP1-AS1	WNT1-inducible signaling pathway protein 1-antisense transcript 1
WT1	Wilms tumor protein 1
XbaI	*Xanthomonas badrii* I
XIC	X inactivation centre
XIST	X-inactive specific transcript
ZEB2	zinc finger E-box binding homeobox 2
ZEB2-AS1	zinc finger E-box binding homeobox 2-antisense transcript 1
ZF5	zinc finger protein 5
ZFPM2-AS1	zinc finger protein, FOG family member 2-antisense transcript 1

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Curriculum vitae

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Work Experience and Education

Dec 2014 – Dec 2017 **PhD position**, *Research Training Group GRK1591 project B2, Group of prof. Gekle; Julius Bernstein Institute of Physiology, Medical Faculty*, Halle (Saale), Germany.

Mar 2012 – Feb 2013 **Master thesis**, *Group of Dr. Vugrek; Laboratory for translational medicine, The Ruđer Bošković Institute*, Zagreb, Croatia.

Oct 2010 – Feb 2013 **Master degree in molecular biology**, *Faculty of Science, University of Zagreb*, Zagreb, Croatia.

Aug – Oct 2011 **IAESTE Internship**, *Group of prof. Jeltsch; Laboratory for Biochemistry and cell biology, Jacobs University*, Bremen, Germany.

Sep – Oct 2010 **IAESTE Internship**, *Group of Dr. Scharf; Laboratory for Molecular biology, Clinic and Policlinic for otorhinolaryngology, University Greifswald*, Greifswald, Germany.

Jul 2005 – Sep 2010 **Bachelor degree in molecular biology**, *Faculty of Science, University of Zagreb*, Zagreb, Croatia.

Oct 2001 – Jun 2005 **High school title: Nurse**, *Medical high school*, Karlovac, Croatia.

Workshops

12 Dec 2014 **Writing papers and theses in the life sciences**, *by Prof. Martin K. Wild*, Halle (Saale), Germany.

29–30 Sep 2016 **Critical reasoning and logic**, *by Dr. Malte Engel*, Halle (Saale), Germany.

19 Apr 2016 **Good scientific practice**, *by Prof. Wahle*, Halle (Saale), Germany.

Awards

2012 Special Rector's award for participating in organization of student project "The night of biology"

Publications

2017 **Polovic, Mirjana** and Dittmar, Sandro and Hennemeier, Isabell and Humpf, Hans-Ulrich and Seliger, Barbara and Fornara, Paolo and Theil, Gerit and Azinovic, Patrick and Nolze, Alexander and Köhn, Marcel and Schwerdt, Gerald and Gekle, Michael (2017) "Identification of a novel lncRNA induced by the nephrotoxin ochratoxin A and expressed in human renal tumor tissue." Cellular and Molecular Life Sciences, Vol. 75 (12), pp 2241–2256, doi:10.1007/s00018-017-2731-6.

Oral presentations

14 Nov 2017 **"WISP1-AS1 influence on cellular functions"**, *6. Thesis committee meeting*, Institute for Biochemistry and Biotechnology, Halle (Saale), Germany.

10 Nov 2017 **"WISP1-AS1, a long noncoding RNA involved in renal toxicity and cancer"**, *The 4th Congress for Doctoral students*, Halle (Saale), Germany.

04 Apr 2017 **"WISP1-OT2 influence on cellular functions"**, *5. Thesis committee meeting*, Institute for Biochemistry and Biotechnology, Halle (Saale), Germany.

03 Nov 2016 **"Orientation of the long noncoding RNA, WISP1-OT2"**, *4. Thesis committee meeting at the Retreat of the Research Training Group GRK1591*, Wyndham Garden Hotel, Quedlinburg, Germany.

12 Apr 2016 **"Characteristics and functions of WISP1-OT2 in HEK293T cells"**, *3. Thesis committee meeting*, Institute for Biochemistry and Biotechnology, Halle (Saale), Germany.

08 Apr 2016 **"Long non-coding RNAs: from discovery to function"**, *Retreat of the Research Training Group GRK1591*, Wyndham Garden Hotel, Quedlinburg, Germany.

03 Nov 2015 **"Characterizing long non-coding RNA, lncWISP1"**, *2. Thesis committee meeting*, Institute for Biochemistry and Biotechnology, Halle (Saale), Germany.

20 Apr 2015 **"Characterizing the effects of overexpression of the long non-coding RNA, lncWISP1, in HEK293T cells"**, *1. Thesis committee meeting*, Berghotel zum Edelacker, Freyburg/Unstrut, Germany.

Poster presentations

02 Jun 2017 **226 "Function of the long noncoding RNA, WNT1-inducible signaling pathway protein 1-overlapping transcript 2 (WISP1-OT2) in human renal cells"**, *The 22nd Annual Meeting of the RNA Society*, Prague, Czech Republic.

16 Mar 2017 **A01-2 "Function of the long noncoding RNA, WNT1-inducible signaling pathway protein 1-overlapping transcript 2 (WISP1-OT2) in human renal cells"**, *The 96th annual meeting of the German Physiology Society*, Greifswald, Germany.

04 Mar 2016 **P17-14 "Function of an alternative transcript of the Wnt1-inducible signaling pathway protein 1 gene in human renal cells"**, *The 95th annual meeting of the German Physiology Society*, Lübeck, Germany.

Declaration / Erklärung

Hereby I sincerely declare, according to § 5 of the Doctoral Regulations of the Faculty of Natural Sciences I at the Martin Luther University Halle-Wittenberg, that I have prepared and wrote this thesis on my own and without external help. *Hiermit, erkläre ich, gemäß § 5 der Promotionsordnung der Naturwissenschaftlichen Fakultät I der Martin-Luther-Universität Halle-Wittenberg, dass ich die vorliegende Dissertation eigenständig und ohne fremde Hilfe angefertigt und angeschreiben habe.*

The submitted written version of the thesis corresponds to that on the electronic storage medium. *Die eingereichte schriftliche Fassung der Arbeit entspricht der auf dem elektronischen Speichermedium.*

In order to carry out the work and to prepare the thesis, I have used only the sources and resources that I specified. *Zur Durchführung der Arbeiten und zur Vorbereitung der Dissertationsschrift wurden nur die von mir angegebenen Quellen und Hilfsmittel verwendet.*

The statements and thoughts taken directly or indirectly from external sources have been identified as such in the thesis. *Die aus fremden Quellen direkt oder indirekt übernommenen Aussagen und Gedanken wurden in der Dissertationsschrift als solche kenntlich gemacht.*

Furthermore, I also declare that this thesis does not exist in the same or similar form in another examination procedure.
Im Übrigen erkläre ich auch, dass diese Dissertation nicht in gleicher oder ähnlicher Form in einem anderen Prüfungsverfahren vorliegt wurde.

I hereby declare that I did not undertake any previous doctorate attempt, and that no doctorate attempt is initiated at another scientific institution. *Hiermit erkläre ich, dass ich keinen vorausgegangenen Promotionsversuch unternommen habe, und dass kein Promotionsversuch an einer anderen wissenschaftlichen Einrichtung läuft.*

I hereby declare that I am neither convicted, nor that preliminary investigations are pending against me. *Hiermit erkläre ich, dass ich weder vorbestraft bin, noch dass gegen mich Ermittlungsverfahren anhängig sind.*

Place (*Ort*), Date (*Datum*)	Signature of the applicant (*Unterschrift der Antragstellerin*)

Acknowledgements

I would like to thank everyone who made this thesis possible and who supported me along the way. Especially thanks to:

My supervisors from the Julius Bernstein Institute. Dr. Gerald Schwerdt for his exceptional support and help with laboratory instruments and protocols. Prof. Michael Gekle for the opportunity to perform my doctoral thesis in his work group. I am thankful for his guidance and the constant willingness to listen and understand the issues and trying to find the best solutions. My supervisor from the Faculty of Natural Sciences I, Prof. Dr. Stefan Hüttelmaier, for the help and support with the project, especially for the valuable ideas and comments.

Reviewers that found time to read my thesis.

Dr. Zdravko Lorković, whom I had the privilege to meet at the University of Zagreb as a lecturer of the subject 'RNA biology'. Thanks to his exceptional approach to holding lectures and including students to actively participate in lectures. My love for RNA biology stems from that time.

Technicians for help with handling materials and methods and support in obtaining results. They always tried to find a good answer on thousand questions I had.

Prof. Claudia Grossmann for help with ordering primers and for the cheerful talks in the kitchen while preparing coffee or tea.

Dr. Alexander Nolze for the initial data processing of RNA sequencing.

Marie-Christin Schulz for keeping me company in the lab outside the work context, for all the ideas and jokes about work, interests and life.

The colleagues who helped with settling in Halle. Likewise, all the colleagues and GRK members who had interesting and helpful ideas and comments about my project, who were interested in my work, especially in the never ending story of Northern blot.

Dr. Selma Gago Zachert for the help in establishing Northern blot method, useful discussions and appreciated comments. I feel grateful for many small details about Northern blot that she taught me.

Anne Baude, for administrative help regarding the project, especially for calculating Tagesgeld.

Marcel Köhn for enormous help with my project, tips on performing experiments and analyzing the data. Also, I want to apologize for all the times I misspelled your name.

DFG for financing the project.

The PhD Network of the MLU for providing German courses, excursions and social events. I really enjoyed Christmas parties! Through German courses I met many friends and very nice people from all around the world.

The greatest gratitude goes to Willem for love and support along the way, listening about all the complaints and problems. For finding right, calming words when I needed compassion, and for making jokes to cheer me up.

My friends and family for the long distance love and support, Skype calls and chats. Additionally, thanks to Jennifer for proofreading my thesis. I am sure I forgot some "the" somewhere.