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„Natural products and their putative effect on *NORAD*"

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## ABSTRACT

While the majority of the human genome is transcribed, only a small fraction of these RNA transcripts is ultimately translated into proteins. The plethora of non-coding RNAs have increasingly attracted attention, with the group of long non-coding RNAs (lncRNAs) emerging as particularly interesting, because of their functions in embryonic development, differentiation and disease. *Noncoding RNA activated by DNA damage* (*NORAD*) is an abundant and conserved lncRNA that has recently been uncovered as a regulator of genomic stability, mitochondrial function and aging in mammalian cells and tissues. Furthermore, *NORAD* expression can be increased by cellular stressors, including DNA damage and hypoxia, although the exact involvement of the lncRNA and the functional consequences of its induction in these and other stress response pathways, are not yet understood.

Natural products have been investigated for a myriad of human diseases and have emerged as modulators of lncRNAs in several human disease models. Therefore, seven natural products, all known to exhibit anti-cancer, antioxidant, anti-aging and longevity effects among others, were selected to explore a potential effect on *NORAD* in this thesis.

While *NORAD* +/+ and *NORAD* -/- cells did not differ in viability or intracellular ROS production upon treatment with the selected compounds, qRT-PCR analysis revealed an induction of *NORAD* by curcumin at 10 µM, rapamycin at 5 µM and 1 µM and resveratrol at 100 µM. This induction may likely be a result of cellular stress caused by the compounds, as we suggest a possible correlation between an increase in oxidative stress and an increase in *NORAD,* as well as a potential connection of *NORAD* induction to p53. These findings therefore emphasize the role of *NORAD* in cellular stress response and may provide further lines of investigation into the mechanisms and outcomes of *NORAD* modulation by the hereby tested compounds, as well as the involvement of the lncRNA in other stress response pathways and whether these could be modulated by natural products.

## ZUSAMMENFASSUNG

Während der Großteil des menschlichen Genoms transkribiert wird, wird letztendlich nur ein kleiner Teil dieser RNA-Transkripte in Proteine übersetzt. Die Vielzahl an nicht-kodierender RNAs erlangt zunehmend an Beachtung, wobei die Gruppe der langen nicht-kodieren RNAs (long non-coding RNA, lncRNAs) aufgrund ihrer Funktionen bei der Embryonalentwicklung und verschiedenen Krankheiten besonders hervorsticht. *Noncoding RNA activated by DNA damage* (*NORAD*) ist eine häufig vorkommende und konservierte lncRNA, die erst kürzlich als Regulator von genomischer Stabilität, Mitochondrienfunktion und des Alterns in Säugetierzellen und -geweben beschrieben wurde. Darüber hinaus ist bekannt, dass die Expression von *NORAD* durch zelluläre Stressoren wie DNA-Schäden und Hypoxie stimuliert wird, wobei die genaue Beteiligung der RNA und die funktionellen Konsequenzen ihrer Induktion in diesen und anderen Aspekten der zellulären Stressantwort noch nicht bekannt sind.

Naturstoffe werden für eine Vielzahl menschlicher Krankheiten untersucht und haben sich in mehreren menschlichen Krankheitsmodellen als Modulatoren von lncRNAs erwiesen. Aus diesem Grund wurden für diese Arbeit sieben Naturstoffe ausgewählt, die vor allem für ihre antikarzinogenen, antioxidativen und lebensverlängernden Effekte bekannt sind, um einen möglichen Effekt auf *NORAD* zu untersuchen.

Während in Bezug auf Lebendzellanteil und intrazellulärer ROS-Produktion keine Unterschiede zwischen *NORAD* +/+ und *NORAD* -/- Zellen nach Behandlung mit den ausgewählten Stoffen festzustellen waren, ergab die qRT-PCR-Analyse eine Induktion von *NORAD* durch Curcumin (10 µM), Rapamycin (5 µM und 1 µM) und Resveratrol (100 µM). Diese Induktion tritt möglicherweise als Folge von intrazellulärem Stress auf, der durch Behandlung mit den Testsubstanzen verursacht wird. Eine mögliche Korrelation zwischen einem Anstieg in oxidativem Stress und einem Anstieg von *NORAD*, sowie ein potentieller Zusammenhang zwischen *NORAD*-Induktion und p53, konnte in den Untersuchungen aufgezeigt werden. Die Ergebnisse dieser Arbeit unterstreichen daher die Rolle von *NORAD* in der zellulären Stressantwort und liefern Ansatzpunkte für weitere Untersuchungen hinsichtlich der Mechanismen und Konsequenzen der *NORAD*-Modulation durch Naturstoffe.

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# 1 INTRODUCTION

## 1.1 Long non-coding RNAs

Most of the human genome has been shown to be transcribed into corresponding RNA transcripts. However, only a small fraction of these RNAs is ultimately translated into proteins, leaving the majority of transcripts without any coding potential. [1] These non-coding RNAs (ncRNAs) comprise many classes of RNA molecules, including housekeeping ncRNAs, such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), as well as regulatory ncRNAs. [2,3] Regulatory ncRNAs can further be categorized into short and long non-coding RNAs, defined by a sequence length shorter or longer than 200 nucleotides, respectively. [3,4] Among short ncRNAs are microRNAs (miRNAs), small-interfering RNAs (siRNAs) and piwi-associated RNAs, which have known functions in regulating gene expression on a post-transcriptional level. [1,4] In contrast, long non-coding RNAs (lncRNAs) represent a very heterogenous family of RNA molecules, for most of which the exact biological function remains elusive. [1,4] They are roughly characterized by a sequence length of more than 200 nucleotides and the absence of any relevant protein-coding potential or open reading frame (ORF). [1,5]

LncRNAs can be located between protein-coding genes, originate from promotor or enhancer regions, or overlap other genes in a sense or antisense direction. [1,6] Like mRNAs, they are transcribed by RNA polymerase II and can be 5' capped, 3' polyadenylated and spliced, but are much lower in abundance and evolutionarily less conserved than protein-coding genes. [1,2] This is, partly, the reason why the vast majority of lncRNAs remains uncharacterized, even though human cells are thought to harbor tens of thousands of them. [1,5] Furthermore, it has been suggested that some lncRNAs may even encode small proteins or originate from genes that yield both protein-coding and non-coding transcripts. [1,7]

While their molecular mechanisms are diverse, it has been widely proposed that lncRNAs exert important gene regulatory functions in mammalian cells, which they can perform either in *cis* or in *trans*. [1,2,6] *Cis*-acting lncRNAs regulate the expression of neighboring genes, which they can do by specifically guiding chromatin-modifying proteins to certain loci, thereby

either activating or repressing their transcription. It has also been proposed that it is not the lncRNA itself, but rather the process of its transcription and/or splicing that exerts this regulatory function. Another potential mechanism suggests that it is (the location of or promotor elements within) the DNA sequence of the lncRNA that harbor the *cis*-regulatory properties, irrespective of whether the gene is transcribed or not. Apart from regulating gene expression in *cis*, lncRNAs can perform a variety of functions in *trans*. These include modulation of chromatin structure to regulate transcription of distant genes, reorganization of nuclear structures, as well as regulating the activity of other RNAs and/or proteins to which they bind, often in a stoichiometric manner. As for their subcellular localization, lncRNAs can be found in the nucleus and the cytoplasm. While the majority localize to the nucleus to perform their regulatory activities in *cis*, *trans*-acting lncRNAs can also operate in the cytoplasm. [1,6]

In comparison to protein-coding mRNAs, lncRNAs are often expressed in tissue-, development-, and disease-specific patterns and have been proposed to play key roles in physiological processes, such as development, differentiation and tissue homeostasis. [7-9] Moreover, lncRNAs have been implicated in a variety of human diseases, including cancer, cardiovascular and neurodevelopmental disorders and could serve as valuable biomarkers, prognostic markers and therapeutic drug targets. [1,7,10]

## *1.2 NORAD*

*Noncoding RNA activated by DNA damage* (*NORAD*), is a *trans*-acting, predominantly cytoplasmatic lncRNA that can be distinguished from other members of its family in that it is both highly abundant in mammalian cells and well-conserved among mammalian species. [1,11] In humans, *NORAD* is present in cell lines and tissues throughout the entire body, at an unusually high amount of approximately 500-1,000 copies per cell, resembling the expression levels of housekeeping mRNAs, such as actin B (ACTB). The highest levels of *NORAD* are found in the brain. [5,11]

*NORAD* (or *LINC00657*) is a transcript of 5.3 kb in length and is located on chromosome 20q11.23. It originates from a strong promotor, is unspliced and most likely transcribed by RNA polymerase II because of its 3' polyadenylation and the presence of H3K4me3-modified histones at the transcription start site. [11-13] Moreover, *NORAD* has been shown to have no substantial protein-coding potential or open reading frame (ORF) and is therefore highly unlikely to produce any functional proteins. [11,12]

Similar to other lncRNAs, *NORAD* has a repeated nature. Its sequence contains distinct elements that recur multiple times throughout the entire transcript and have been described as either 12 repeating units of approximately 300 nucleotides (nt) each, or as 5 larger regions of about 400 nt each, referred to as *NORAD* domains. [6,11,12,14] Within these repetitive elements lie many of the conserved regions of *NORAD*, including a U-rich sequence, a short hairpin of 4 base pairs (bp), a long hairpin of 8-9 bp, an A/G-rich element, as well as binding sites for the RNA-binding proteins PUMILIO 1 and PUMILIO 2 and other RNA-binding proteins. [11,14-16] As for the subcellular localization of the lncRNA, it has been demonstrated that while 80-90% of the transcript reside in the cytoplasm, a small fraction may also remain in the nucleus. [16]

### 1.2.1 Functions of *NORAD*

Recent research has uncovered that *NORAD* is essential for maintaining genomic stability and mitochondrial function in mammalian cells and tissues, as well as for preventing the development of a degenerative phenotype resembling premature aging in mice. Furthermore, *NORAD* has been proposed to be involved in cellular stress response pathways, particularly to DNA damage and hypoxia. [5,11]

#### 1.2.1.1 Preservation of genomic and mitochondrial stability

The predominantly cytoplasmatic localization of the lncRNA, as well as the abundance of conserved elements within its sequence, have led to the proposal that *NORAD* functions as a binding platform for RNA-binding proteins, thereby mediating the formation of

ribonucleoprotein (RNP) complexes. [11] Indeed, one of the mechanisms through which *NORAD* exerts its functions is by binding to and buffering the activity of mammalian RNA-binding proteins PUMILIO1 (PUM1) and PUMILIO2 (PUM2) in the cytoplasm. [5,11] PUM proteins are part of the family of Pumilio and Fem3 binding factor (PUF) proteins, which is deeply conserved among eukaryotic species. PUM proteins bind specifically to the eight-nucleotide sequence UGUANAUA, which is typically found in the 3' UTRs of mRNAs and has been referred to as the PUMILIO response element (PRE). [5,11] Upon binding, they actively repress the translation of these mRNAs through increasing their deadenylation and accelerating decay. [5,6] These targets include genes involved in the regulation of the cell cycle, mitosis, DNA replication and repair, as well as mitochondrial biogenesis and homeostasis. [5,11]

PREs can not only be located in 3' UTRs, but also in 5' UTRs of mRNAs, in coding sequences and in non-coding RNAs. They are most highly enriched in 3' UTRs of mRNAs and in non-coding RNAs, which is also where PUM proteins have been proposed to have the most powerful regulatory effects. [17] The lncRNA *NORAD* possesses an unusually high amount of 15 conserved PREs per transcript, through which it can bind PUM1/2 and prevent them from repressing the translation of PRE-containing mRNAs. This negative regulation of PUM proteins by *NORAD* has been demonstrated to be required for maintaining genomic and mitochondrial stability in mammalian cells. [5,6,11] Accordingly, loss of *NORAD* leads to PUM1/2 hyperactivity, resulting in dramatic chromosomal instability, abnormal mitosis and mitochondrial dysfunction in human cells. [5,11] Inactivation of the mouse ortholog *Norad* revealed the same consequences in the animals, as well as characteristic features associated with premature aging, such as increased graying of fur and alopecia, weight loss, kyphosis, aging-related skeletal muscle and neurological abnormalities and a lower survival rate. [5]

Interestingly, PUF proteins can be found widely across eukaryotic species, whereas *NORAD* orthologs seem to appear specifically within mammals, indicating that mammalian PUMILIO levels must be kept within a tight range. [5,11] This is corroborated by studies stating that a decrease in PUMILIO activity can be just as harmful as an increase, leading to behavioral problems, abnormal neurogenesis and neurodegeneration, as well as aneuploidy in mammalian cells and tissues. [5,6,11]

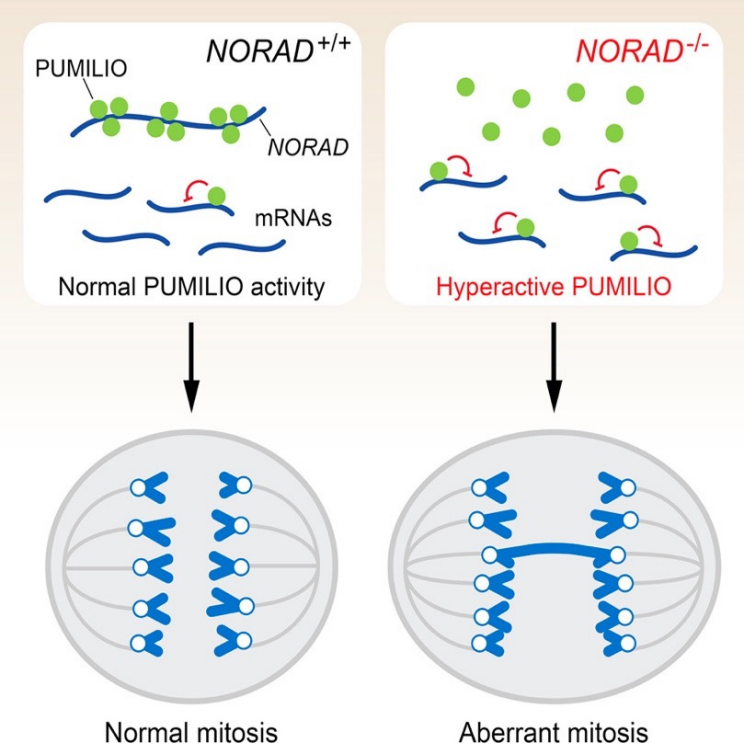



**Figure 1.** Schematic representation of the *NORAD*-PUMILIO axis (adopted from Lee *et al*., 2016 [11])

Furthermore, it has recently been uncovered that SRC-associated in mitosis of 68 kDa (SAM68), an abundant RNA-binding protein involved in a variety of cellular processes, is needed for efficient regulation of PUM2 by *NORAD*. [5,14,18] SAM68 is able to bind both *NORAD* and PUM2 in human cells, thereby facilitating the interplay between the two and the subsequent *NORAD*-mediated PUM2 repression. Loss of SAM68 also leads to chromosomal instability, independently of *NORAD,* suggesting that it is also needed to keep PUMILIO activity in check. SAM68 binds to the A/G-rich regions within the repetitive elements of *NORAD*, which show a rate of conservation similar to those of *NORAD* PREs. [14]

Another proposed mechanism for the maintenance of genomic stability by *NORAD* includes its interaction with RNA-binding motif protein X-linked (RBMX) in the nucleus. [13,15,16] RBMX binds specifically to a longer than 800-nt element at the 5’ end of *NORAD*, which is conserved among mammals and is the strongest RBMX-binding site in the transcriptome. [15] It has been suggested that the *NORAD*-RBMX interaction mediates the formation of an RNP

complex by facilitating the binding of RBMX to a variety of proteins essential to DNA replication or repair, including topoisomerase I (TOP1) among others. [15,16] The hereby generated RNP complex has been referred to as the *NORAD*-activated ribonucleoprotein complex 1 (NARC1). [15] While it has been proven on more than one occasion that RBMX does indeed bind to *NORAD*, results on whether the *NORAD*-RBMX interaction is essential to maintaining genomic stability have been controversial. [5,15,16] However, it remains possible, that *NORAD* exerts different functions in different subcellular compartments, including the interaction with PUM1/2 in the cytoplasm and with RBMX in the nucleus and that these pathways do not have to exclude each other. [15,16]

#### 1.2.1.2 Involvement in cellular stress responses

In addition to its functions described above, *NORAD* has been proposed to play a considerable role in the response to cellular stressors, with its expression being upregulated by DNA damage and hypoxia. [5,11] In human cells and in mice, *NORAD* is induced upon treatment with DNA-damaging agents doxorubicin or camptothecin in a p53-dependent manner. No substantial p53-binding site was found within or near the *NORAD* promotor, since however an increase in *NORAD* levels upon DNA damage was only detected in p53 +/+ but not in p53 -/- cells, the lncRNA is thought to be an indirect target of p53. [11] Moreover, *NORAD* deletion did not affect the p53-mediated DNA damage response, suggesting that the lncRNA is not needed for this pathway. [11] Additionally, it has been shown that *NORAD*, along with other highly conserved lncRNAs, is induced by hypoxia (0.2% $O_2$) in endothelial cells. [11,19] The functional outcome of *NORAD* upregulation under these conditions, as well as the effects on its downstream targets, such as PUM1/2, or the possible involvement of the lncRNA in other stress response pathways, remain to be explored. [5,11]

### 1.2.2 *NORAD* in human disease

As is the case for several other lncRNAs, dysregulation of *NORAD* has been implicated in different steps of carcinogenesis, including proliferation, invasion, epithelial-mesenchymal transition (EMT), apoptosis and metastasis. [7,13] *NORAD* can be upregulated or downregulated and act as either an oncogene or a tumor suppressor gene in human cancers. In some cancer types, the lncRNA functions as an oncogene when upregulated and is associated with larger tumor size, higher tumor stage, poor prognosis and poor overall survival. These include breast, lung, pancreatic, colorectal, bladder and cervical cancer among others. In other cancer types, such as hepatocellular carcinoma, *NORAD* levels correlate inversely with malignancy, thus making it a tumor suppressor. [13]

Apart from cancer, dysregulation of lncRNAs has been implicated in other age-associated diseases, such as neurodegenerative disorders. [1,20] In this context, *in vitro* experiments have shown that low levels of *NORAD* can worsen, whereas high levels can protect against Parkinson's disease, however, this has yet to be demonstrated *in vivo*. [21] Interestingly, it is known that *NORAD* levels decrease in the human brain upon aging, particularly in the subependymal zone, a region responsible for regenerating adult brain cells, suggesting that the lncRNA may protect from aging in humans as well. [20,22]

## 1.3 Natural products and lncRNAs

Natural products have been revealed as modulators of lncRNAs in a number of chronic human disease models. These include predominantly cancer models, but also osteoarthritis, rheumatoid arthritis and nonalcoholic fatty liver disease models. Phytochemicals can up- or downregulate lncRNAs, by targeting transcription factors, kinases, other enzymes or tumor-associated miRNAs. In cancer models, they may further promote chemo- and radiosensitization of tumor cells. Natural products that have been demonstrated to modulate lncRNAs in these models include curcumin, resveratrol, epigallocatechin gallate, paclitaxel, genistein, silibinin, among others. [9]

## 1.4 Natural products tested in this work

### 1.4.1 Acetylsalicylic acid

Acetylsalicylic acid (aspirin) is a derivative of natural salicylates present in the willow bark and acts as a prodrug to salicylate, to which it is rapidly metabolized by hepatic, intestinal and plasma esterases *in vivo*. [23-26] As a non-steroidal anti-inflammatory drug (NSAID), aspirin inhibits cyclooxygenase 1 (COX1) and COX2 and thereby prevents the synthesis of prostaglandins and thromboxane, resulting in anti-platelet, anti-inflammatory, analgesic and antipyretic effects. [24] Additional mechanisms of action have been proposed for the drug, including the repression of NF-kB signaling, activation of AMPK and the inhibition of acetyltransferase EP300, thereby inducing autophagy. [23,26] Low-dose aspirin is used for the secondary prevention of cardiovascular diseases, whereas its use for primary prevention is still regarded as controversial, due to its side effects, e.g. increased risk of bleeding. [24,25] Furthermore, regular intake of low-dose aspirin has displayed chemopreventive properties against several human cancers, including breast, colorectal, ovarian and prostate cancer. [24,25] Additionally, aspirin has been shown to prolong lifespan in Drosophila, C. elegans and mice, probably by inducing autophagy. [26,27] It has also been proposed to prevent pre-eclampsia and Alzheimer's disease and has been studied in age-related hearing loss as well as for antiviral activities. [24] Moreover, *in vitro* studies have revealed the ability of aspirin to modulate lncRNAs in several cancer cell lines, such as inducing the lncRNA OLA1P2 or repressing the lncRNA H19, both events contributing to its cancer-preventive effects. [28,29]

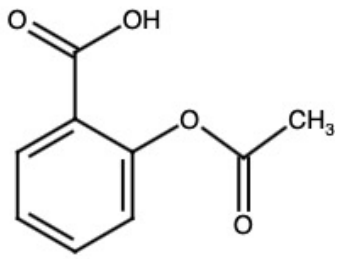



**Figure 2.** Chemical structure of acetylsalicylic acid

### 1.4.2 Curcumin

Curcumin is a dietary polyphenol found in turmeric (*Curcuma longa* L.) that has been widely used in food coloring, as a spice and in traditional medicine. [30,31] A multitude of health benefits have been ascribed to the compound, including anticarcinogenic, anti-inflammatory, antioxidant, antimicrobial, antidiabetic, anti-aging, antiandrogenic and neuroprotective properties. [30,32-34] Curcumin has been proposed to modulate a variety of cellular signaling pathways *in vitro* by influencing transcription factors, kinases and inflammatory mediators, including p53, NF-κB, MAPK, Akt, Nrf2, JAK/STAT among others. [31-34] In cancer cells, the polyphenol has been shown to inhibit proliferation and induce apoptosis, both via the intrinsic and extrinsic pathway. [32,34] Furthermore, it has been proposed that curcumin modulates a number of lncRNAs in cancer cells and models, by downregulating oncogenic and upregulating tumor-suppressive lncRNAs. [9] Many clinical trials in humans have investigated the effects of curcumin *in vivo* against various diseases, including cancer, diabetes, cardiovascular, neurological, skin, inflammatory and infectious diseases. [33-35] However, its application *in vivo* is limited by its poor pharmacokinetic profile, particularly its water insolubility, poor bioavailability and extensive metabolism. [30,33-35] Structural modification as well as the use of new delivery systems, such as nanoparticles, liposomes or formulations with proteins or various oils, have been developed to overcome these barriers. [30,33,34]

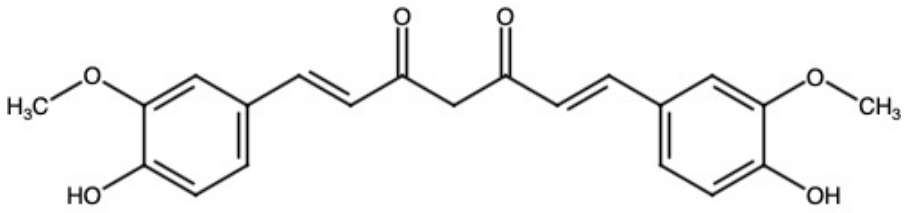



**Figure 3.** Chemical structure of curcumin

### 1.4.3 Epicatechin

(-)-Epicatechin and (+)-epicatechin, along with the other catechins (-)-catechin and (+)-catechin, are flavonoids that can be found in numerous fruits, vegetables and plant products, including tea, red wine and cocoa, whereas apples, cherries, plums and chocolate are particularly rich in epicatechin. [36,37] Several human clinical trials and animal studies have revealed the protective effects of catechins against cardiovascular diseases, and other age-related pathologies, including neurodegenerative diseases and cancer. [23,37] Many of these effects have been ascribed to the powerful antioxidant capacities of catechins, which they can mediate directly by scavenging ROS and chelating metal ions, or indirectly, by upregulating antioxidant enzymes such as superoxide dismutase (SOD) or catalase (CAT) or inhibiting prooxidant enzymes, including NADPH oxidase. [37] Furthermore, catechins have been proposed to modulate oxidative stress- and inflammation-related signaling pathways, such as NF-KB signaling, and stimulate NO production by activating eNOS in endothelial cells, the latter resulting in the vasodilative effects observed in animals and humans. [36-38] Additionally, catechin has been shown to extend lifespan of nematodes, independently of its antioxidative effects. [23] Also, the bioavailability of catechins seems to be favorable and they can exert their protective effects at relatively low plasma concentrations which are achievable by consuming catechin-rich foods. [23,36]

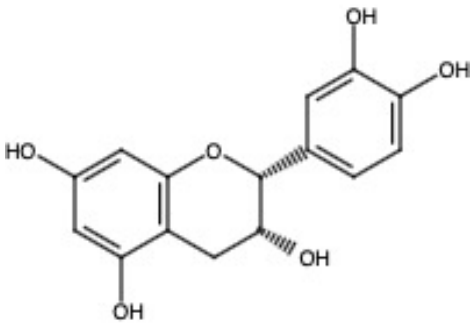



**Figure 4.** Chemical structure of (-)-epicatechin

### 1.4.4 Metformin

Metformin (1,1-dimethylbiguanide) is derived from natural guanidines present in the French lilac (*Galega officinalis*) and is an oral antidiabetic currently used as a first-line treatment for type 2 diabetes mellitus. [23,39-41] Apart from its blood glucose-lowering activity, the drug has revealed anticarcinogenic, cardio- and vasoprotective and life-prolonging properties. [39,41,42] Many of its effects are thought to be mediated through the inhibition of complex I of the mitochondrial respiration chain and subsequent AMPK activation, which leads to reduced gluconeogenesis, lipogenesis and protein synthesis in hepatocytes, as well as stimulation of eNOS in endothelial cells, which account for the antidiabetic and cardioprotective effects of metformin, respectively. [39,41,43] AMPK activation also plays a role in the anti-aging effects of the drug, through inhibition of mTOR signaling, whereas metformin can exert its lifespan-extending properties in an AMPK-independent fashion as well, e.g. through induction of autophagy. [23,42] The anti-cancer effects of the compound are most likely due to suppression of cell proliferation by inhibiting mTOR signaling, but also due to reducing the growth-promoting effects of insulin and insulin-like growth factor 1 (IGF-1), and through modulating inflammatory processes by lowering NF-κB signaling. [39,42] Furthermore, research has shown that metformin modulates a number of lncRNAs in *in vitro* and *in vivo* models, including downregulating lncRNA TUG1, which is pathologically upregulated in atherosclerosis, or upregulating lncRNA H19, which is decreased in patients with polycystic ovary syndrome (PCOS), as well as repressing the lncRNA Dreh in skeletal muscle cells, thereby enhancing glucose uptake. [44-46] However, much of the exact molecular mechanism of metformin is still unknown, with further research ongoing. [40,42,43,47]

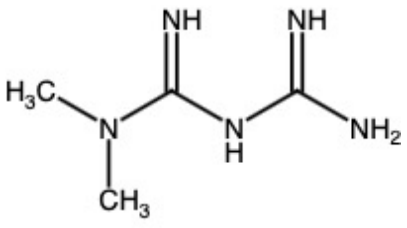



**Figure 5.** Chemical structure of metformin

### 1.4.5 Rapamycin

Rapamycin (sirolimus) is a natural macrolide produced by *Streptomyces hygroscopicus* that displays antifungal, anti-carcinogenic, immunosuppressive, cardioprotective, neuroprotective as well as lifespan-extending properties. [23] Mechanistically, rapamycin forms a complex with FK506-binding protein 12 (FKBP12), which then inhibits mammalian target of rapamycin (mTOR), a kinase that is integral to two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). [48,49] mTORC1 regulates cell growth and proliferation through boosting anabolic processes, such as protein, lipid and nucleotide synthesis by responding to nutrient, energy and growth signals. [49,50] mTORC1 dysregulation has been linked to several human diseases, including cancer, neurodegeneration, type 2 diabetes mellitus as well as to aging. [51] mTORC2 also plays a role in cell survival, through activation of several kinases, including Akt. [50,52] While mTORC1 is acutely sensitive to inhibition by rapamycin, mTORC2 is only inhibited by long-term exposure to the macrolide. [50,52] Most of the beneficial effects of rapamycin, including its anti-cancer and anti-aging properties, have been associated with the inhibition of mTORC1, whereas many of the side effects of chronic rapamycin treatment, such as insulin resistance, diabetes and immunosuppression have been linked to mTORC2 inhibition. [50,52] To avoid some of these negative effects in the use of rapamycin as a longevity agent, different approaches have been proposed to selectively inhibit mTORC1, including intermittent administration of the drug. [52]

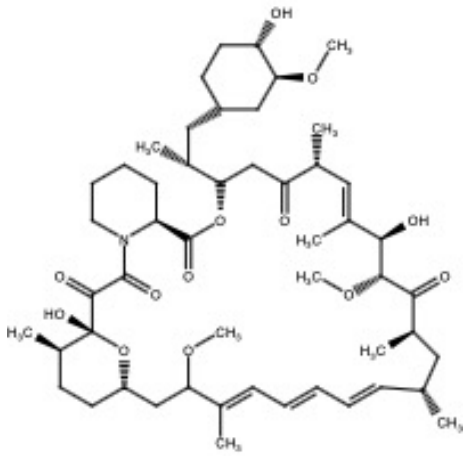


**Figure 6.** Chemical structure of rapamycin

### 1.4.6 Resveratrol

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a natural polyphenol that is found in numerous plant foods, including grapes, berries and peanuts, as well as in red wine and tea. [53-55] It exists as both *cis* and *trans* isomers, whereas *trans*-resveratrol seems to be the more abundant form in nature. [55] The effects of resveratrol are pleiotropic, ranging from anti-cancer, antioxidant and anti-inflammatory activities to neuroprotective and anti-aging properties. [55] Many *in vitro* studies have revealed the anticarcinogenic effects of resveratrol, which it can exert through a variety of different mechanisms, including cell cycle arrest, apoptosis induction, modulation of CYP450 enzymes and upregulation of the tumor suppressor p53. [53,54]. Similar to other polyphenols, resveratrol can function as a powerful antioxidant, however, depending on cell type and concentration, it can also exert prooxidant effects, the latter contributing to its anticarcinogenic potential. [54] Furthermore, resveratrol has anti-inflammatory properties, improves insulin sensitivity, regulates blood pressure and blood fat levels, through modulating a variety of molecules, including COX1 and COX2, inducible NO synthases (iNOS) and AMPK, all of which underlie its protective effects against cardiovascular disease and metabolic syndrome. [23,56] Additionally, the polyphenol has been shown to prolong lifespan in a variety of *in vitro* and animal models, mainly through the activation of sirtuin 1 (SIRT1), thereby imitating the positive effects of caloric restriction. [23,55,56] Among the many molecular targets of resveratrol are also a number of lncRNAs, which are modulated by the polyphenol in various cancer cell lines, including colorectal cancer, lung cancer and glioma cells. [9] In spite of its therapeutic potential demonstrated in *in vitro* studies, its low bioavailability due to extensive phase II metabolism in the liver and intestine, limit its application in vivo. [53,54,56]

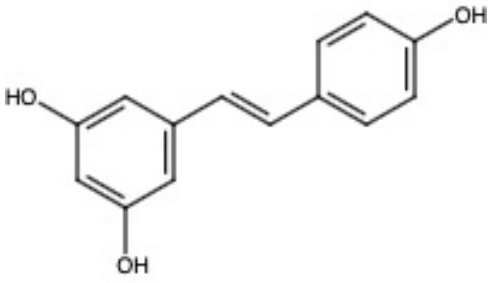



**Figure 7.** Chemical structure of *trans*-resveratrol

### 1.4.7 Spermidine

Spermidine is a natural polyamine found in numerous plant foods, including soybeans, nuts, mushrooms, green peas, broccoli, mango and wheat germ. [57,58] Apart from these food sources, spermidine can be generated via cellular biosynthesis and by intestinal microorganisms. [57] Polyamines are essential for cell growth and proliferation through stabilization of DNA and RNA and numerous other mechanisms, but their levels decrease with age in human tissues, as well as in model organisms. [57] Accordingly, spermidine supplementation has been shown to extend lifespan in flies, nematodes, and mice. [57] These anti-aging properties have been proposed in humans as well, by protecting against age-related pathologies, such as cardiovascular disease, cognitive impairment and cancer through a plethora of effects, including antioxidant, anti-inflammatory, immune-stimulating, tumor-suppressive and neuroprotective properties. [57,59] On a molecular level, spermidine induces autophagy, mainly by inhibiting several acetyltransferases, such as EP300, which mostly accounts for its life-prolonging effects. [23,57] However, autophagy-independent mechanisms have been proposed as well, including indirect antioxidant activities and increased NO production by enhancing arginine bioavailability. [57] In spite of its beneficial effects, elevated spermidine levels have also been linked to human diseases, including cancer, stroke and renal failure, suggesting that spermidine concentrations should be maintained within an optimal range. [57]

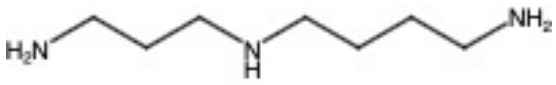



**Figure 8.** Chemical structure of spermidine

## 1.5 Aims of this work

*Noncoding RNA activated by DNA damage* (*NORAD*) is a highly abundant and conserved long non-coding RNA (lncRNA) which is involved in regulating genomic stability, mitochondrial function and aging in mammalian cells and tissues. It is furthermore known that *NORAD* is induced upon cellular stressors, such as DNA damage and hypoxia. [5,11]

Since a variety of phytochemicals are known to modulate lncRNAs in cellular models of human disease, the aim of this work was to investigate whether selected natural products exert any effects on *NORAD*. The compounds chosen for this work all exhibit pleiotropic effects, including anti-aging and life-prolonging properties, as well as anti-cancer activities. The goal of this study was to investigate whether any of these effects were mediated through *NORAD* and whether the compounds affect *NORAD* expression levels and what the functional outcomes of such a modulation would be.

# 2 MATERIALS AND METHODS

## 2.1 HCT116 cells

Human colon carcinoma HCT116 wild type (*NORAD* +/+) and HCT116 *NORAD* -/- cells were kindly provided by Ass.-Prof. Dr. Florian Kopp (Department of Pharmaceutical Chemistry, University of Vienna) and are characterized by an epithelial morphology and adherent growth. HCT116 *NORAD -/-* cells were generated by inactivating *NORAD* in HCT116 cells via transcription-activator-like effector nucleases (TALEN)-mediated insertion of a transcriptional stop element within the first 300 nucleotides of the *NORAD* gene, followed by puromycin selection, as described by Lee *et al*. [5]

### 2.1.1 Culture

HCT116 and HCT116 *NORAD -/-* cells were cultured in McCoy's 5A Medium with L-Glutamine, supplemented with penicillin/streptomycin and 10% heat-inactivated FBS in 75 $cm^2$ cell culture flasks at 37°C and 5% $CO_2$. Cells were subcultured every two to three days, upon reaching 80-90% confluence, which was assessed under a microscope. Before starting the subculturing process, growth medium, PBS and trypsin/EDTA were pre-warmed to 37°C for 30 min. Following the removal of the old medium, cells were washed with 12 mL PBS, trypsinized with 3 mL trypsin/EDTA and incubated for 2 min at 37°C and 5% $CO_2$. The sides of the flask were tapped gently to help detach the cells from the bottom surface and trypsin digestion was stopped by resuspending the cells in 7 mL fresh growth medium. 1 mL of the cell suspension was subsequently removed for cell counting and viability measurement, using a ViCell™ hemocytometer. Upon counting, 12 mL fresh growth medium and 4 x $10^6$ or 2 x $10^6$ cells (for passaging every 2 or 3 days respectively) were transferred to new 75 $cm^2$ flask and incubated at 37°C and 5% $CO_2$.

### 2.1.2 Freezing and thawing

For freezing, HCT116 and HCT116 *NORAD -/-* cells were harvested and counted as described above. After counting, cells were centrifuged at 1400 rpm for 4 min (Thermo Scientific Heraeus™ Multifuge™ 3L-R). The supernatant was decanted, and the cell pellet was resuspended in 6 mL of cold freezing medium (growth medium supplemented with 5% DMSO as a cryopreservation agent). The cell suspension was subsequently aliquoted into cryovials which were first kept on ice and then stored in a polystyrene box at -80°C overnight. This ensured that the cells were frozen slowly. After freezing overnight, the frozen cells were transferred to liquid nitrogen (-196°C) for long-term storage.

For thawing, the cryovials with the frozen cells were removed from liquid nitrogen storage and quickly thawed in a 37°C water bath until there was just a small clump of ice left in the vial. In the laminar flow hood, the thawed cells were suspended in 25 mL warm growth medium and incubated in a 75 $cm^2$ flask at 37°C and 5% $CO_2$.

**Table 1.** Cell culture materials

| **Name** | **Ingredients** | | **Provider** |
|---|---|---|---|
| Growth medium | McCoy's 5A with L-Glutamine | 500 mL | Lonza |
| | FBS | 50 mL | Gibco |
| | Penicillin | 100 U/mL | Lonza |
| | Streptomycin | 100 µg/mL | Lonza |
| PBS | NaCl | 7.2 g | Carl-Roth |
| | $Na_2HPO_4$ | 1.48 g | Carl-Roth |
| | $KH_2PO_4$ | 0.43 g | Carl-Roth |
| | $ddH_2O$ | 1000 mL | |
| Trypsin/EDTA | Trypsin | 0.5 g | Gibco |
| | $Na_2$- EDTA | 0.2 g | Gibco |
| | PBS | 1000 mL | |
| DMSO | | | Gibco |

## 2.2 Compounds

Acetylsalicylic acid, curcumin, epicatechin, metformin, rapamycin, resveratrol and spermidine were dissolved in DMSO at a concentration of 100 mM each, aliquoted to 100 µL and further diluted to working concentrations of 10 mM, 7 mM, 5 mM, 3 mM, 1 mM, 0.5 mM and 0.1 mM, which were stored at -80°C. For all experiments, these stock solutions were diluted 1:1000 with fresh growth medium to achieve final concentrations of 10 µM, 7 µM, 5 µM, 3 µM, 1 µM, 0.5 µM and 0.1 µM per well.

## 2.3 Resazurin conversion assay

The resazurin conversion assay was performed to assess the effects of the compounds on cell viability. Resazurin is a cell-permeable redox dye that, in metabolically active cells, can be reduced to resorufin, a pink and fluorescent product. The amount of resorufin positively correlates with the number of viable cells and can be quantified via fluorescence measurement. [60]

For the assay, HCT116 and HCT116 *NORAD -/-* cells were seeded in 96-well plates at a density of 2 x $10^3$ cells/well in regular growth medium and incubated for 48 h at 37°C and 5% $CO_2$. Following the aspiration of the old medium, cells received 100 µL of fresh growth medium and were treated with 100 µL of the compounds (10 µM, 7 µM, 5 µM, 3 µM and 1 µM in DMSO), vehicle control (DMSO 0.1%) or positive control (digitonin 50 µg/mL) and incubated for 72 h. After that, the old medium was removed, and cells were incubated with 150 µL resazurin (10 µg/mL in growth medium). Fluorescence was measured at an emission wavelength of 580 nm after 2 h, using a Tecan Spark® microplate reader.

In order to analyze the effect of repeated treatment with selected compounds on cell viability, HCT116 and HCT116 *NORAD -/-* cells were stimulated with curcumin, metformin and spermidine for 3 consecutive days. To this end, 2 x $10^3$ cells were seeded in 96-well plates and incubated for 24 h at 37°C and 5% $CO_2$. Then, the old medium was aspirated, cells received 100 µL of fresh growth medium and were treated with 100 µL of the compounds

(10 µM, 5 µM, 1 µM, 0.5 µM and 0.1 µM in DMSO), vehicle control (DMSO 0.1%) or positive control (digitonin 50 µg/mL) and incubated for 24 h. Then, cells were treated as indicated for the second time, incubated for 24 h, then treated as indicated for the third time and again incubated for 24 h. Before each treatment, the old medium was aspirated, and cells received 100 µL of fresh growth medium. Following 3 days of treatment, the old medium was removed and 150 µL resazurin (10 µg/mL in growth medium) were added to each well, and fluorescence was measured at 580 nm after 2 h, using a Tecan Spark® microplate reader.

**Table 2.** Test compounds, substances and positive controls

| **Name** | **Stock solution** | **Use** | **Provider** |
|---|---|---|---|
| Acetylsalicylic acid | 100 mM in DMSO | Test compound | Sigma-Aldrich |
| Curcumin | 100 mM in DMSO | Test compound | Tocris Bioscience |
| Epicatechin | 100 mM in DMSO | Test compound | Sigma-Aldrich |
| Metformin | 100 mM in DMSO | Test compound | Sigma-Aldrich |
| Rapamycin | 100 mM in DMSO | Test compound | Sigma-Aldrich, Santa Cruz Biotechnology |
| Resveratrol | 100 mM in DMSO | Test compound | Sigma-Aldrich |
| Spermidine | 100 mM in DMSO | Test compound | Sigma-Aldrich |
| Digitonin | 5 mg/mL in EtOH | Positive control for resazurin conversion assay | Sigma-Aldrich |
| Doxorubicin | 1 mM in $H_2O$ | Positive control for qRT-PCR | Sigma-Aldrich |
| Resazurin sodium salt | 0.1 mg/mL in PBS | Resazurin conversion assay | Sigma-Aldrich |

## 2.4 Measurement of reactive oxygen species (ROS)

The influence of the compounds on the production of ROS in HCT116 and HCT116 *NORAD -/-* cells was analyzed via flow cytometry, using 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) as an indicator of cellular ROS. $H_2$DCF-DA is a cell-permeable nonfluorescent dye, that, once internalized, is hydrolysed to the nonpermeable 2',7'-dichlorodihydrofluorescein ($H_2$DCF) by intracellular esterases. $H_2$DCF is easily oxidized by ROS, generating the highly fluorescent 2',7'-dichlorofluorescein (DCF), which can be quantified fluorometrically and is directly proportional to the amount of intracellular ROS. [61]

To detect ROS levels in HCT116 and HCT116 *NORAD -/-* cells, 400 x $10^3$ cells were seeded in in 6-well plates and incubated for 24 h at 37°C and 5% $CO_2$. Following the renewal of the medium, cells were treated with all compounds (10 µM in DMSO), vehicle control (DMSO 0.1%) or resveratrol (100 µM in DMSO), which was used as a control for increased ROS production, for 24 h. Then, the old medium was removed, cells were washed with 2 mL PBS, trypsinized with 300 µL trypsin/EDTA and resuspended in 700 µL fresh growth medium. The cell suspension was subsequently divided into FACS tubes, centrifuged at 1100 rpm for 5 min (Thermo Scientific Heraeus™ Multifuge™ 3L-R), resuspended in 500 µL $H_2$DCF-DA (20 µM in PBS/BSA 2%) or 500 µL PBS/BSA 2% and incubated in the dark for 30 min. Afterwards, cells were centrifuged at 1100 rpm for 5 min (Thermo Scientific Heraeus™ Multifuge™ 3L-R), washed with 500 µL PBS/BSA 2%, once again centrifuged at 1100 rpm for 5 min (Thermo Scientific Heraeus™ Multifuge™ 3L-R) and resuspended in 300 µL PBS/BSA 2%. Intracellular ROS levels were quantified by measuring fluorescence of DCF at an emission wavelength of 525 nm, using a BD FACSCalibur™ flow cytometer (Flh1 channel). All samples were measured in duplicates (with or without $H_2$DCF-DA) to determine the autofluorescence of the cells, which was subtracted from cells treated with $H_2$DCF-DA during data analysis. All data were analyzed using the CellQuest™ Pro Software.

ROS production upon repeated treatment with selected compounds was also measured via flow cytometry. 400 x $10^3$ cells were seeded in three 6-well plates and incubated for 24 h at 37°C and 5% $CO_2$. Then, cells on all three plates were treated with curcumin, metformin, spermidine (10 µM in DMSO), rapamycin (5 µM and 1 µM in DMSO), resveratrol (100 µM in DMSO) or vehicle control (DMSO 0.1%) for 24 h. After that, cells on plate number 1 were harvested and ROS levels following 24 h compound treatment were analyzed as described above. Cells on plate number 2 and plate number 3 were treated as indicated for the second time and incubated for 24 h. Then, cells on plate number 2 were harvested for analysis of ROS levels following 48 h compound treatment, and cells on plate number 3 were treated for the third time and analyzed for ROS production after a total of 72 h of treatment. Before each treatment, growth medium was aspirated from the wells and compound-free medium was added.

**Table 3.** Buffers and solutions for ROS measurement

| **Name** | **Ingredients/stock solutions** | | **Provider** |
|---|---|---|---|
| FACS buffer pH 7.37 | NaCl | 8.12 g | Carl-Roth |
| | $Na_2HPO_4$ | 2.35 g | Carl-Roth |
| | $KH_2PO_4$ | 0.26 g | Carl-Roth |
| | KCl | 0.28 g | Carl-Roth |
| | LiCl | 0.43 g | Carl-Roth |
| | $NaN_3$ | 0.20 g | Carl-Roth |
| | $Na_2EDTA$ | 0.36 g | Carl-Roth |
| | $ddH_2O$ | 1000 mL | |
| PBS/BSA 2% | BSA | 2 g | Carl-Roth |
| | PBS | 100 mL | |
| $H_2DCF$-DA | 20 mM in DMSO (stock solution) | | Invitrogen |

## 2.5 RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed to validate HCT116 *NORAD* +/+ (wild type) and HCT116 *NORAD* -/- (knockout) cells and to analyze the effects of selected compounds on *NORAD* expression.
For the validation of HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells, 400 x $10^3$ cells were seeded in 6-well plates, incubated for 24 h and total RNA was isolated, using the peqGOLD Total RNA Kit (VWR Life Science). Briefly, cells were washed with 1 mL PBS, lysed with 350 µL of lysis buffer and total RNA was extracted according to the manufacturer's instructions. The isolated RNA was quantified and checked for purity via the A260/A280 ratio (≥2.0) using a NanoDrop 2000 spectrophotometer and stored at –80°C. 681 ng of RNA were subsequently transcribed to cDNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). The generated cDNA samples were stored at -80°C until further use.

To analyze the effects of selected compounds on *NORAD* expression, 400 x $10^3$ cells were seeded in 6-well plates, and then treated with curcumin, metformin, spermidine (10 µM in DMSO), rapamycin (5 µM and 1 µM in DMSO) resveratrol (100 µM) or vehicle control (DMSO 0.1%) for one, two or three days. Doxorubicin (1 µM in $H_2O$) was used as a positive control for *NORAD* upregulation. Total RNA was isolated with the peqGOLD Total RNA Kit (VWR Life Science) as described above. 1 µg of RNA was transcribed to cDNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™).
*NORAD* expression levels were quantified via qPCR on a LightCycler®480, using the Luna® Universal qPCR Master Mix (New England BioLabs®Inc.) and appropriate primers (see table 5), which were kindly provided by Ass.-Prof. Dr. Florian Kopp (Department of Pharmaceutical Chemistry, University of Vienna). Actin B and GAPDH were used as reference genes and *NORAD* expression was normalized to expression levels of actin B or GAPDH using the ΔCt method.

**Table 4.** Thermal cycling conditions for cDNA synthesis

| **Step** | **Temperature** | **Time** |
|---|---|---|
| Step 1 | 25 °C | 10 min |
| Step 2 | 37 °C | 120 min |
| Step 3 | 85 °C | 5 min |
| Step 4 | 4 °C | ∞ |

**Table 5.** Primers for qPCR

| **Gene** | **Direction** | **Sequence 5' to 3'** |
|---|---|---|
| Actin B | Forward primer | CCAACCGCGAGAAGATGA |
| Actin B | Reverse primer | CCAGAGGCGTACAGGGATAG |
| GAPDH | Forward primer | AGCCACATCGCTCAGACAC |
| GAPDH | Reverse primer | GCCCAATACGACCAAATCC |
| NORAD | Forward primer | TGATAGGATACATCTTGGACATGGA |
| NORAD | Reverse primer | TGGACACATCTGCATACATCTCT |

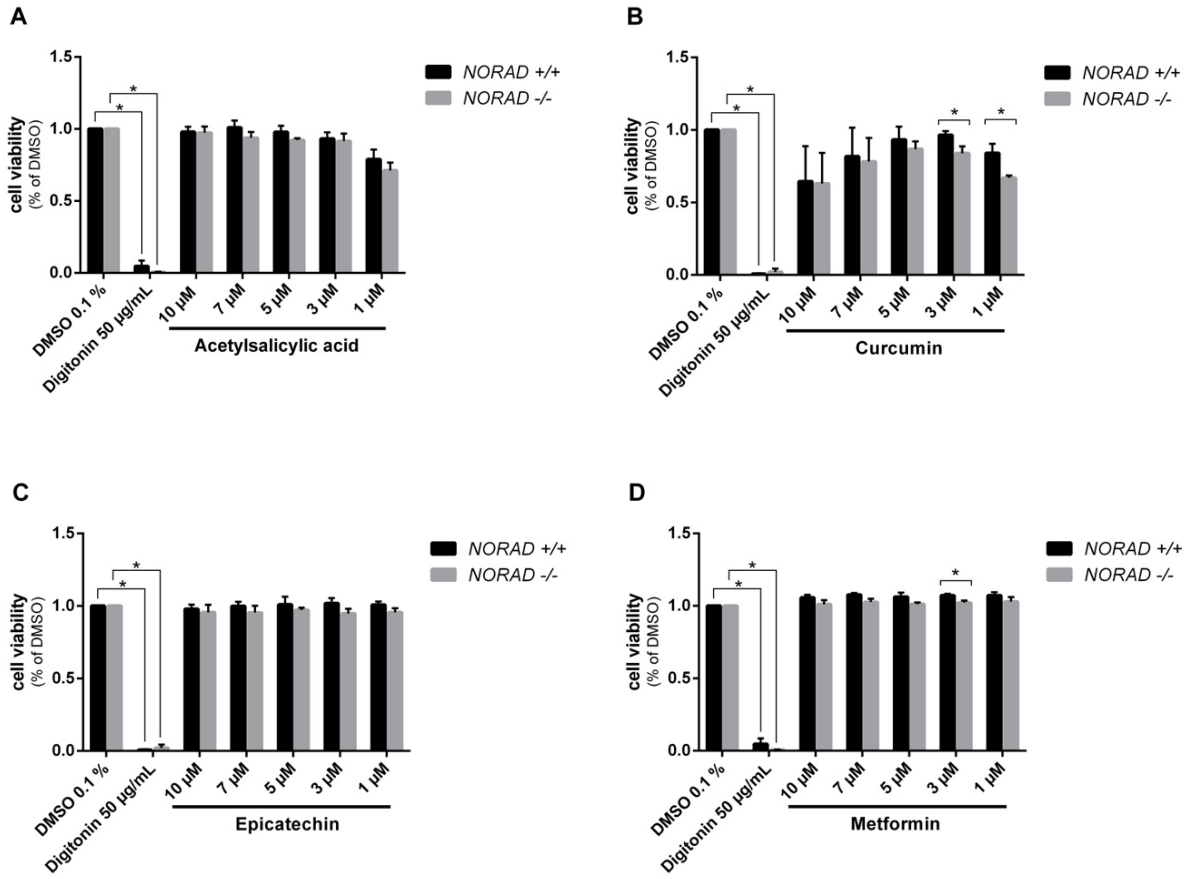
**Table 6.** qPCR conditions

| **Step** | **Temperature** | **Time** |
|---|---|---|
| Pre-Incubation | 95 °C | 1 min |
| Amplification | 95 °C<br>60 °C | 15 sec<br>30 sec |
| Melting | 95 °C<br>55 °C<br>98 °C | 15 sec<br>30 sec |
| Cooling | 40 °C | 10 sec |

# 3 RESULTS

## 3.1 *NORAD* +/+ and *NORAD* -/- cells do not markedly differ in viability upon one-time treatment with natural products

*NORAD* is a highly conserved and abundant lncRNA that functions as a regulator of genomic stability, mitochondrial function and aging in mammalian cells. [5,11] Several natural products, known for their anti-cancer, anti-aging and longevity effects among others, have been selected to investigate a potential effect on *NORAD* in HCT116 *NORAD* +/+ and *NORAD* -/- cells. While a comparison of cell viability upon treatment with compounds, using the resazurin conversion assay, did not reveal any obvious difference between *NORAD* +/+ and *NORAD* -/- cells for acetylsalicylic acid, epicatechin and resveratrol, it revealed a small effect for curcumin, metformin, rapamycin and spermidine, although this is likely not biologically significant (**Figure 9A-G**).



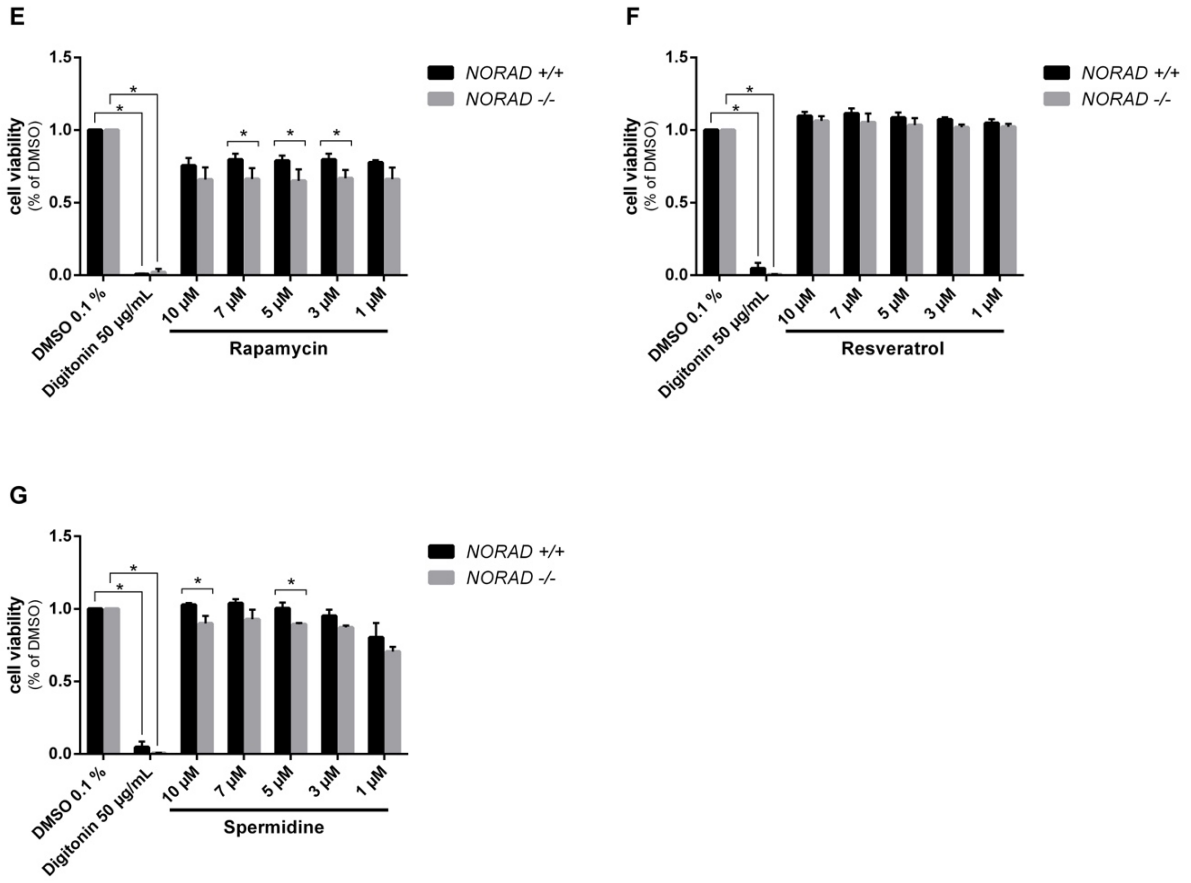



**Figure 9. (A-G) Effects of compounds on cell viability.**
HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells were treated with the indicated compounds (10 µM, 7 µM, 5 µM, 3 µM, 1 µM in DMSO), vehicle control (DMSO 0.1%) or positive control (digitonin 50 µg/mL). Cell viability was determined after 72 h, using the resazurin conversion assay. Cells were incubated with resazurin (10 µg/mL) and fluorescence was measured at 580 nm after 2 h (n = 3 biological replicates). Data are presented as mean ± SD, and p-values were calculated using Student's t-test. * $p < 0.05$.

## 3.2 *NORAD* +/+ and *NORAD* -/- cells do not differ in viability upon repeated treatment with natural products

Because some of the tested natural products are known to be extensively metabolized (both *in vitro* and *in vivo*), with the metabolites being less efficacious than the parent compounds, *NORAD* +/+ and *NORAD* -/- cells were treated with selected compounds for 72 h (3 treatments in 24 h intervals) in order to ensure that they were exposed to the parent compounds long enough for them to exert their potential effects. [30,36,53]

While cell viability decreased in comparison to one-time treatment, no obvious difference between wild type and knockout cells could be observed **(Figure 10A-C)**. It was therefore concluded that cell viability is not an adequate readout to determine any potential effects of these compounds on *NORAD*.

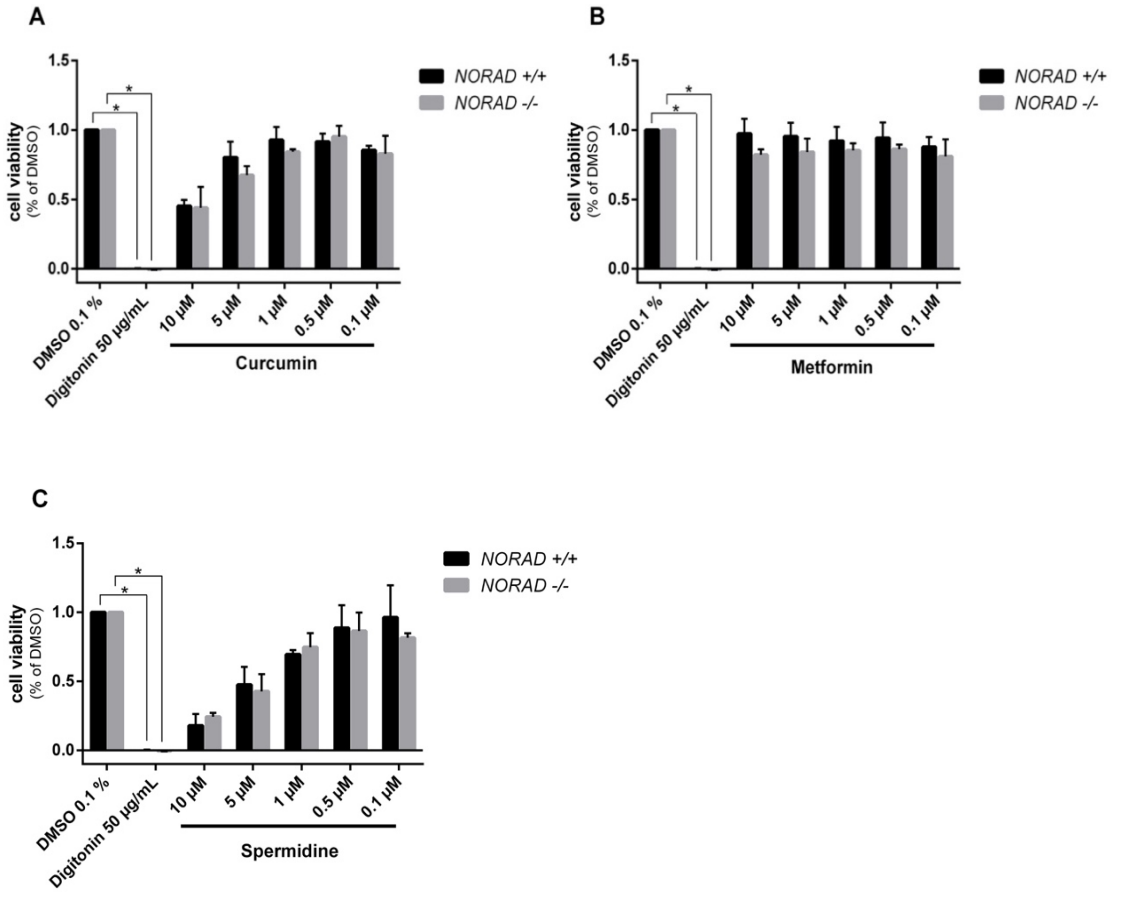



**Figure 10. (A-C) Effects of repeated treatment on cell viability.**
HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells were treated with the indicated compounds (10 µM, 5 µM, 1 µM, 0.5 µM and 0.1 µM in DMSO), vehicle control (DMSO 0.1%) or positive control (digitonin 50 µg/mL) 3 times in 24 h intervals. Growth medium was aspirated from the wells and replaced with compound-free medium before each treatment. Cell viability was determined using the resazurin conversion assay. Cells were incubated with resazurin (10 µg/mL) and fluorescence was measured at 580 nm after 2 h (n = 3 biological replicates). Data are presented as mean ± SD, and p-values were calculated using Student's t-test. * $p < 0.05$.

## 3.3 Intracellular ROS levels do not differ in *NORAD* +/+ and *NORAD* -/- cells upon treatment with compounds

Research has shown that *NORAD* is essential for maintaining mitochondrial function, with its loss resulting in increased ROS levels and reduced respiration in HCT116 cells. [5] This could be validated by comparing basal ROS production in *NORAD* +/+ and *NORAD* -/- cells (**Figure 11A**). Because many of the tested natural products are known to display antioxidant, and some also prooxidant effects, we wondered if this was, in some part, related to *NORAD*. [30,36,54,57] To this end, intracellular ROS levels upon treatment with the selected natural products were quantified in *NORAD* +/+ and *NORAD* -/- cells via flow cytometry. However, although the compounds did indeed display antioxidant tendencies, the analysis revealed no significant difference between *NORAD* +/+ and *NORAD* -/- cells (**Figure 11B**).

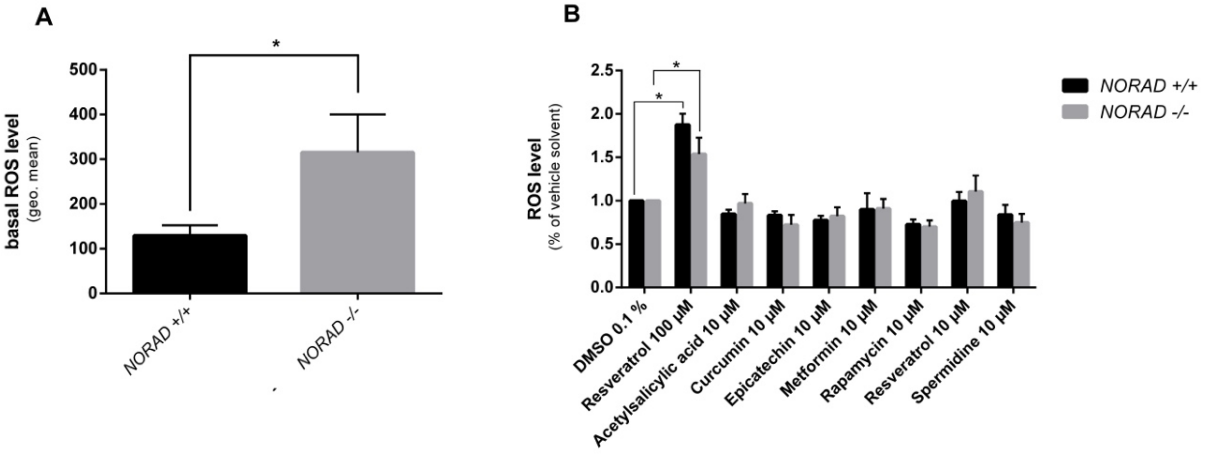



**Figure 11. Analysis of intracellular ROS levels via flow cytometry.**
**(A)** Basal ROS levels in HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells, analyzed via flow cytometry (n = 11 biological replicates). **(B)** Intracellular ROS levels after treatment with compounds. HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells were treated with the indicated compounds (10 µM in DMSO), vehicle control (DMSO 0.1%) or resveratrol (100 µM in DMSO), which was used as a control for increased ROS production, for 24 h. Intracellular ROS levels were analyzed via flow cytometry by measuring DCF-derived fluorescence after incubation of cells with $H_2$DCF-DA for 30 min (n = 3-6 biological replicates). Data are presented as mean ± SD in **A-B**, and p-values were calculated using Student's t-test. * $p < 0.05$.

Furthermore, *NORAD* +/+ and *NORAD* -/- cells were treated with selected compounds one (24 h), two (48 h) or three (72 h) times in 24 h intervals to analyze the effects of prolonged treatment on ROS production (**Figure 12A-C**). The analysis revealed no significant difference in ROS levels between wild type and knockout cells upon treatment with curcumin, metformin, rapamycin or spermidine. Interestingly, resveratrol at 100 µM, which was used as a control for increased ROS production, generated significantly more oxidative stress in wild type than in knockout cells, when compared to the respective negative control (DMSO 0.1%), both upon one-time and repeated treatment (**Figure 11B, Figure 12A-C**). Since the same effect was observed upon treatment with doxorubicin at 1 µM (**Figure 12D**), which is known to upregulate *NORAD* in HCT116 cells at this concentration [11], and a slight tendency to the mentioned effect could be observed upon treatment with curcumin, metformin and spermidine as well (after 72 h; **Figure 12C**), we wondered if this could indicate a potential connection to *NORAD*.

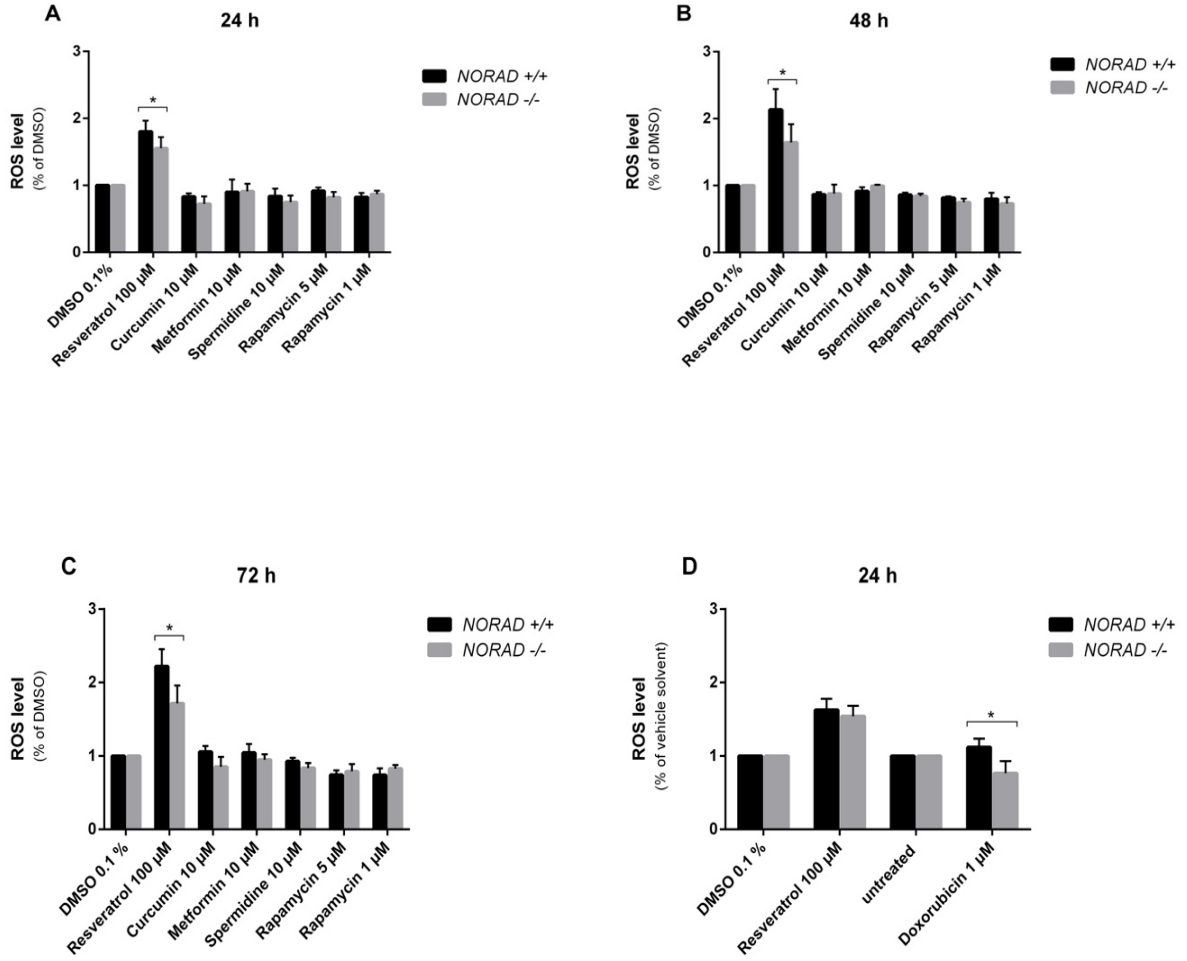


**Figure 12. Effects of repeated treatment on intracellular ROS levels.**
**(A-C)** HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells were treated with the indicated compounds (10, 5 or 1 µM in DMSO), vehicle control (DMSO 0.1%) or resveratrol (100 µM in DMSO) as a control for increased ROS production one (24 h), two (48 h) or three (72 h) times in 24 h intervals. Intracellular ROS levels were analyzed via flow cytometry by measuring DCF-derived fluorescence after incubation of cells with $H_2$DCF-DA for 30 min (n = 3-9 biological replicates). **(D)** HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells were treated with doxorubicin (1 µM in $H_2O$) after 24 h. Intracellular ROS levels were analyzed via flow cytometry (n = 5 biological replicates). All data were normalized to the respective vehicle control (compounds to DMSO, doxorubicin to untreated cells). Data are presented as mean ± SD in **A-D** and p-values were calculated using Student's t-test. * $p < 0.05$.

## 3.4 Curcumin, rapamycin and resveratrol increase *NORAD* expression levels in HCT116 cells

In order to elucidate a potential effect of selected compounds on *NORAD* expression, *NORAD* levels were quantified via qRT-PCR in HCT116 wild type cells upon 1, 2 or 3 days of treatment. The analysis revealed a significant increase in *NORAD* levels upon treatment with resveratrol, with a 2-fold increase after 1 day, augmenting to a 6-fold increase after 2 and 3 days of treatment. While treatment with curcumin revealed no increase in *NORAD* levels after 24 h, it did upregulate the lncRNA after 48 h and even more after 72 h, suggesting a dose-dependent effect. Rapamycin revealed an increase in *NORAD* levels already after 24 h, which climbed further after 48 h and decreased again after 3 days of treatment (**Figure 13B-D**).
It is known that *NORAD* is induced in response to cellular stressors, such as DNA-damaging agent doxorubicin, which was used as a positive control in these experiments. [11] Given that curcumin (10 µM) significantly decreased viability after 3 days (**Figure 10A**), but not so much after 1 day of treatment (**Figure 9B**), rapamycin (5 or 1 µM) decreased viability after 1-time treatment irrespective of its concentration (**Figure 9E**) and resveratrol (100 µM) after 1 and 3 days of treatment (data not shown), it is tempting to speculate that these compounds also induce cellular stress that may lead to an increase in *NORAD* levels.

**Figure 13A** shows the results of validation of HCT116 *NORAD* +/+ and HCT116 *NORAD* -/- cells via qRT-PCR.

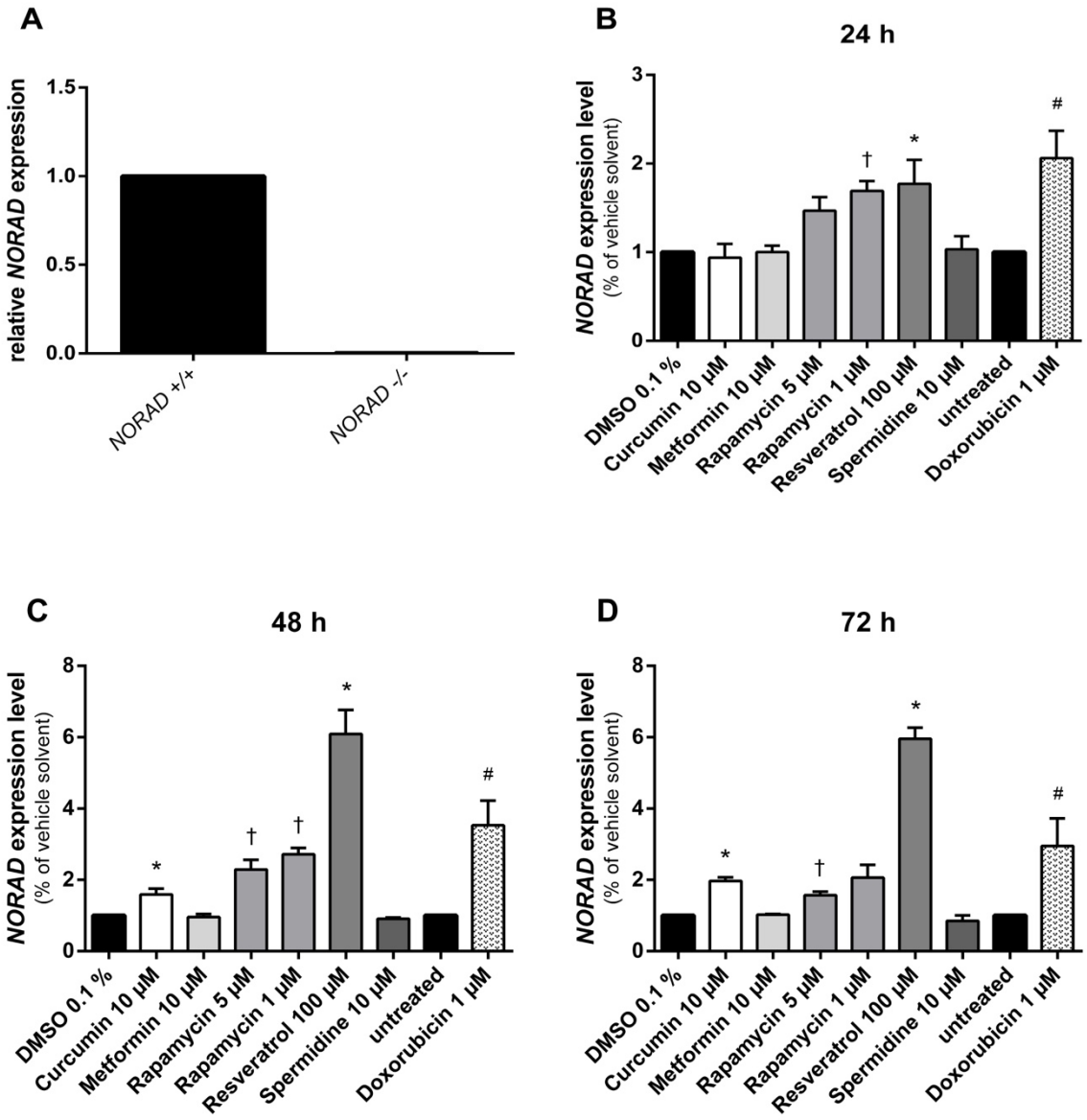



**Figure 13. qRT-PCR analysis.**

**(A)** Validation of HCT116 *NORAD* +/+ and *NORAD* -/- cells. Total RNA was isolated from HCT116 *NORAD* +/+ and *NORAD* -/- cells and *NORAD* expression was quantified via qRT-PCR and normalized to actin B mRNA levels. **(B-D)** Effect of compounds on *NORAD* expression. HCT116 *NORAD* +/+ cells were treated with the indicated compounds (100, 10, 5 or 1 µM in DMSO) or vehicle control (DMSO 0.1%) one (24 h), two (48 h) or three (72 h) times in 24 h intervals. Due to presumed toxicity upon repeated treatment, cells were treated with the positive control (doxorubicin, 1 µM in $H_2O$) only once, 24 h before the respective isolation. Total RNA was isolated after one (24 h), two (48 h) or three (72 h) days of treatment and *NORAD* expression levels were quantified via RT-qPCR. *NORAD* expression was normalized to GAPDH mRNA levels (n= 3-5 biological replicates). Data are presented as mean ± SD in **B-D** and p-values were calculated using Student's t-test for * $p < 0.05$ to DMSO 0.1% and # $p < 0.05$ to untreated cells, and one-way ANOVA, Dunnett for †.

# 4 DISCUSSION

This thesis was conducted in an attempt to investigate whether selected natural products exert any effect on the lncRNA *NORAD*. To this end, all experiments were performed in wild type human colon carcinoma HCT116 (*NORAD* +/+) and HCT116 *NORAD* -/- cells. It is known that *NORAD* is required for maintaining genomic stability and thus ensuring normal mitosis and that the *NORAD*-PUMILIO axis is involved in regulating aging-related phenotypes. [5,11] Furthermore, *NORAD* has been demonstrated to be induced upon cellular stress, including DNA damage and hypoxia. [11] Because many of the tested compounds are known to inhibit cell proliferation on the one hand, but due to the pleiotropic nature of their effects promote longevity through a plethora of mechanisms on the other hand, the question to be addressed by this work was whether any of these effects involve *NORAD*. [23,33,55] While a comparison of cell viability and intracellular ROS production upon treatment with compounds did not show any obvious differences between *NORAD* +/+ and *NORAD* -/- cells, qRT-PCR analysis of *NORAD* expression levels revealed that curcumin, rapamycin and resveratrol induced the lncRNA in HCT116 cells (**Figure 13B-D**).

Since *NORAD* is known to be induced by cellular stressors, it is likely that its increase upon treatment with the above-mentioned compounds is also a result of stress. For instance, 1, 2 or 3 days of treatment with resveratrol (100 µM), which is known to exert prooxidant effects depending on its concentration, elevated not only intracellular ROS production (**Figure 12A-C**), but also *NORAD* expression levels (**Figure 13B-D**), indicating a possible correlation between an increase in oxidative stress and an increase in *NORAD*. [54] Curcumin has been reported to cause oxidative stress in HCT116 cells as well, although at a concentration of 40 µM, which would explain the lack of elevated ROS levels upon treatment at 10 µM (**Figure 12A-C**). [62] However, research has shown that treatment of HCT116 cells with 10 µM curcumin for 48 h, but not for 24 h, resulted in a loss of mitochondrial membrane potential (MMP). [62] Since the present experiments report an increase in *NORAD* expression at this exact time point and concentration (**Figure 13C**) and considering that *NORAD* is required for maintaining MMP, loss of MMP could represent a potential stressor for *NORAD*

induction as well. [5] The previously mentioned co-occurrence of *NORAD* induction and a significant decrease in cell viability could support this theory.

Furthermore, it is known that doxorubicin, a DNA-damaging agent which was used as a positive control for *NORAD* upregulation in these experiments, induces the lncRNA in a p53-dependent manner. [11] Since curcumin and resveratrol have been reported to increase p53 levels in HCT116 cells as well, it would be worth investigating whether their induction of *NORAD* also depends on this tumor suppressor. [62,63] Notably, curcumin has been shown to upregulate p53 levels in HCT116 cells in a concentration-dependent manner and its induction of *NORAD* is also dose-dependent (**Figure 13B-D**), suggesting a potential connection to p53. [64] However, rapamycin does not induce p53, but inhibits cell proliferation by targeting mTORC1, which has been reported to be a downstream target of the tumor suppressor. [66,73] Interestingly, research has proposed that Puf3p, the PUM1/2 ortholog in yeast, is located downstream of the mTOR signaling pathway, with rapamycin treatment limiting Puf3p ability to repress target mRNAs. [65] Given the deep conservation of PUF proteins among eukaryotes, this is likely also the case for mammals, where *NORAD* could represent a missing link between rapamycin treatment and PUF repression, thus providing further evidence for its involvement in the p53/mTOR pathway. [5,11] Moreover, the mTOR pathway is known to respond to nutrient signaling and it has been demonstrated that nutrient deprivation has an inhibitory effect on mTORC1, just like rapamycin. [27,49,67] Considering the possible effect of mTOR inhibition on *NORAD*, starvation might represent another stress factor capable of inducing the lncRNA. In this context, it is noteworthy that hypoxia, another stressor known to upregulate *NORAD* in endothelial cells, has been demonstrated to supress mTORC1 as well. [11,67]

Overall, the exact mechanism(s) of *NORAD* induction by these natural products represent interesting subjects for future research, since not much is known about how *NORAD* expression is regulated in general. Moreover, the cellular consequences of an increase in *NORAD* levels, as well as its potential involvement in other stress response pathways (apart from DNA damage and hypoxia) and whether these can be modulated by natural products are worthy of further investigation. It would furthermore be intriguing to determine PUMILIO activity upon increased *NORAD* levels, particularly because PUM hypoactivity, which is

expected to result from a dramatic induction of *NORAD*, has actually been linked to harmful outcomes. [5,11] Of course, it remains a possibility that the compounds influence *NORAD* independently of PUMILIO, since other functions of *NORAD,* in addition to the regulation of PUMILIO, have been proposed as well. [5]

Apart from anti-cancer effects, curcumin, rapamycin and resveratrol also display antioxidant, anti-aging and life span-extending properties, suggesting a potential use as anti-aging therapeutics. [23] Given the role of the *NORAD*-PUMILIO axis in aging and the observation that *NORAD* levels decrease in the human brain with age, an induction of *NORAD* might certainly be favorable in this context. [5] Since research has shown that the *NORAD*-PUMILIO network is not restricted to a specific cell type, it is likely that the tested compounds would also increase *NORAD* levels in non-cancer cells (e.g. MEFs), which would represent a more suitable cellular model to investigate the effects of aging. [5,11] Specifically, since *NORAD* is essential for mitochondrial function and a decline thereof is generally associated with aging, it would be interesting to see if an increase in *NORAD* by the tested compounds could reduce these aging-associated parameters (e.g. elevated ROS levels, reduced respiration) in a cellular model. [5,68] Because the concentrations that were used for the induction of *NORAD* in HCT116 cells (**Figure 13B-D**) are likely supraphysiologically high, it might be worth investigating if the compounds could achieve the same effect in lower (perhaps more physiologically relevant) concentrations when applied to non-cancer cells.

Additionally, many studies have proposed the synergistic effects of life span-extending agents, which has been suggested for the combination of rapamycin with metformin and resveratrol with spermidine, for instance. [23,57] Combination of rapamycin with resveratrol in cancer cells has revealed synergism as well. [69] In this context, it is further noteworthy that both curcumin and resveratrol have been demonstrated to sensitize cancer cells to chemotherapy, particularly to doxorubicin, gemcitabine and 5-FU. [70-72] Thus, combinatorial treatment with the 3 compounds that induced *NORAD* in these experiments, or combination of one or more of them with anti-cancer drugs (e.g. doxorubicin) in regard to *NORAD* would certainly be of interest as well.

# 5 SUMMARY

Altogether, this work could show that out of several tested natural products, curcumin, rapamycin and resveratrol are capable of increasing the expression of *noncoding RNA activated by DNA damage (NORAD)*, a highly abundant and conserved long non-coding RNA (lncRNA), in HCT116 cells. Whereas the molecular mechanisms and consequences of this modulation remain to be elucidated, this work opens new lines of investigations into the role of natural products as modulators of *NORAD* and other lncRNAs.

# 6 ABBREVIATIONS

| | |
|---|---|
| ACTB | Actin B |
| A/G-rich element | Adenine/Guanine-rich element |
| Akt | Akt Kinase |
| AMPK | 5' Adenosine monophosphate (AMP)-activated protein kinase |
| Bp | Base pair |
| BSA | Bovine serum albumin |
| CAT | Catalase |
| cDNA | Complementary DNA |
| C. elegans | Caenorhabditis elegans |
| COX 1/2 | Cyclooxygenase 1/2 |
| CYP450 | Cytochrome P450 |
| DCF | 2',7'-dichlorofluorescein |
| DMSO | Dimethyl sulfoxide |
| DNA | Desoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EMT | Epithelial-mesenchymal transition |
| eNOS | Endothelial NO synthase |
| EP300 | E1A Binding Protein P300 |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| FKBP12 | FK506-binding protein 12 |
| 5-FU | 5-Fluorouracil |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| HCT116 | Homo sapiens colon colorectal carcinoma ??? |
| $H_2$DCF | 2',7'-Dichlorodihydrofluorescein |
| $H_2$DCF-DA | 2',7'-Dichlorodihydrofluorescein diacetate |
| H3K4me3 | Tri-methylation (me3) at lysine residue 4 (K4) on histone 3 (H3) |
| IGF-1 | Insulin-like growth factor 1 |
| iNOS | Inducible NO synthase |

| | |
|---|---|
| JAK/STAT | Insulin-like growth factor 1 |
| Kb | Kilobase |
| L. | Linné |
| lncRNA | Long non-coding RNA |
| MAPK | Mitogen-activated protein kinase |
| MEFs | Mouse Embyonic Fibroblasts |
| miRNA | MicroRNA |
| MMP | Mitochondrial membrane potential |
| mTOR | Mammalian target of rapamycin |
| mTORC1/2 | Mammalian target of rapamycin complex 1/2 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NARC1 | *NORAD*-activated ribonucleoprotein complex 1 |
| ncRNA | Non-coding RNA |
| NF-kB | Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| *NORAD/Norad* | *Non-coding RNA activated by DNA damage* |
| NO | Nitric oxide |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| NSAID | Non-steroidal anti-inflammatory drug |
| Nt | Nucleotide |
| ORF | Open reading frame |
| PBS | Phosphate buffered saline |
| PCOS | Polycystic ovary syndrome |
| PRE | PUMILIO response element |
| PUF | Pumilio and Fem3 binding factor |
| PUM 1/2 | PUMILIO 1/2 |
| qRT-PCR | Quantitative reverse transcription polymerase chain reaction |
| RBMX | RNA-binding motif protein X-linked |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal RNA |
| RNP | Ribonucleoprotein |
| ROS | Reactive oxygen species |

| | |
|---|---|
| rpm | Revolutions per minute |
| SAM68 | SRC associated in mitosis of 68 kDa |
| siRNA | Small-interfering RNA |
| SIRT1 | Sirtuin 1 |
| SOD | Superoxide dismutase |
| TALEN | Transcription-activator-like effector nucleases |
| tRNA | Transfer RNA |
| TOP I | Topoisomerase I |
| TUG1 | Taurine upregulated gene 1 |
| U-rich sequence | Uracil-rich sequence |
| UTR | Untranslated region |

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