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Title of the proposal:

How Do Small RNAs Impact Gene Expression In Early Embryogenesis?

- **Aim of the Project**

Environmental exposure can affect the parental germ line thereby modulating particular phenotypes in future generations through epigenetic mechanisms. Recent experiments have started to shed light on direct roles for sperm-borne small RNAs, as well as their post-transcriptional modifications, in transferring acquired traits from fathers across generations (Gapp et al, 2014). More specifically, a particular subset of small non-coding RNA molecules, namely tRNA-derived small RNAs (tsRNAs), were reported to relay environmental stress-induced parental phenotypes into the next generation by affecting gene expression patterns in the early embryo (Chen et al, 2016; Sharma et al, 201). Although these seminal experiments uncovered the identity of trait-transmitting molecules, the exact molecular mechanisms as to how tsRNAs mediate transgenerational inheritance are still to be elucidated.

This proposal aims to investigate the molecular mechanisms that govern how tsRNAs contribute to changes in gene expression patterns during embryonic genome activation (EGA) in the early mouse embryo. I hypothesize that not only the sequence information of tsRNAs, but also RNA modifications contribute to their function. To investigate the fate of tsRNAs in early embryo development, I will determine stability as well as localization of specific tsRNAs obtained from wild type and RNA modification mutant sources. To this end, I will purify endogenously produced tsRNAs and chemically label them, followed by microinjections into one cell-stage mouse embryos. Using piezo drilling-aided microinjections, confocal microscopy, fluorescent in situ hybridization and expression analyses I will examine the half-lives, subcellular localization and effects on target gene expression of the injected tsRNAs in early preimplantation embryos. This will allow obtaining a highly resolved picture of the fate of paternally contributed tsRNAs during the course of early mammalian embryogenesis.

- **State of the art**

-Trans-generational inheritance is mediated by epigenetic mechanisms

The concept of the inheritance of acquired traits poses that ancestral experiences (i.e. environmental exposure) can be inherited to offspring to modulate particular phenotypes. This rather controversial idea has been seriously discussed in previous centuries, but has been completely abandoned by modern science, mostly due to the firm establishment of Darwin's theory of evolution and the concomitant disregard of Lamarckism. However, renewed interest in this concept has been rekindled after a number of experiments in various species (ranging from nematodes to mammals) (Rechavi et al, 2014; Carone et al, 2010) showed that particular paternal traits that were acquired in response to specific environmental insults (i.e. susceptibility to radiation, odor sensitivity, mental or diet stresses), are not only transmitted into the next generation but even subsequently propagated by more than one generation (Gapp et al, 2014; Rodgers et al, 2015; Dias et al, 2014; Paris et al, 2015). These findings suggested the existence of molecular mechanisms conveying acquired and non-genetic information to the next generation.

Although earlier experiments could not differentiate between genetic and epigenetic mechanism, the inheritance of acquired traits followed non-Mendelian patterns, indicating epigenetic mechanisms at work. Hence, initially the propagation of aberrant DNA methylation patterns during early development was proposed as a molecular explanation for the inheritance of acquired traits. However, epigenetic reprogramming during early development erases and re-establishes DNA methylation patterns and thus DNA methylation is unlikely to maintain acquired phenotypes throughout generations (Radford et al, 2014). This has prompted the search for other factors that escape developmental reprogramming processes. Various experiments in mammals have now provided proof for a direct role of paternal, sperm-borne RNAs acting as carrier molecules in transferring acquired traits across generations (Grandjean et al, 2015; Chen et al, 2016; Gapp et al, 2014; Sharma et al, 2016). Importantly, stress-induced phenotypes induced by sperm-derived RNAs were stably inherited to subsequent generations, indicating that transgenerational inheritance is likely due to the RNA-mediated induction of additional and stably heritable epigenetic marks (Gapp et al, 2014).

-tRNA fragments are mediators of trans-generational inheritance of acquired traits

Specifically, one class of sperm RNAs: tRNA-derived small RNAs (tsRNAs) appear to carry information to propagate environmentally-induced traits across generations (Chen et al, 2016; Sharma et al, 2016; Zhang et al, 2018). These are small RNAs originating from the 5' or 3' ends of mature tRNAs, which are 32-35 nucleotides in size, and are produced through tRNA cleavage in the anticodon loop, especially in response to stress conditions (Figure 1a) (Yamasaki et al, 2009). tsRNAs constituted the majority of the small RNA load in adult mammalian sperm (Peng et

al, 2014; Chen et al, 2016; Sharma et al, 2016) and their expression changed following environmental stress applied to adult mice and humans (Chen et al, 2016; Donkin et al, 2016), suggesting tsRNAs act as sensors for environmental changes. Zygotic injections of bulk-purified tsRNAs originating from stress-exposed mice successfully recapitulated metabolic phenotypes in the offspring to the same extent as total sperm RNA (Chen et al, 2016; Zhang et al, 2018; Sharma et al, 2016). In addition, injections of specific and highly expressed tsRNAs (i.e. 5' and 3' ends of tRNA-Gly-GCC) into zygotes induced changes in early embryonic gene expression (Sharma et al, 2016; Chen et al, 2016). These seminal experiments clearly indicated that specific tsRNAs play an active role in in the transmission of paternally acquired traits, most likely through effects on early embryonic gene expression regulation.

Importantly, tsRNAs purified from sperm harbored numerous RNA modifications. Particular modifications became elevated upon High Fat Diet (HFD) treatment of adult mice; namely m^1A (1-methyl adenosine), m^5C (5-methyl cytosine) and m^2G (2-methyl guanosine) (Chen et al, 2016; Zhang et al, 2018). These RNA modifications were crucial for the propensity of tsRNAs to mediate trans-generational inheritance of HFD-induced metabolic changes, as zygotic injections of non-modified, synthetic tsRNAs did not recapitulate these metabolic phenotypes (Chen et al, 2016; Zhang et al, 2018). While almost nothing is known about the function of tsRNA modifications, they most likely impact the stability, secondary structure or nucleic acid- or protein-binding capacity of sperm-delivered tsRNAs (FIGURE 1b).

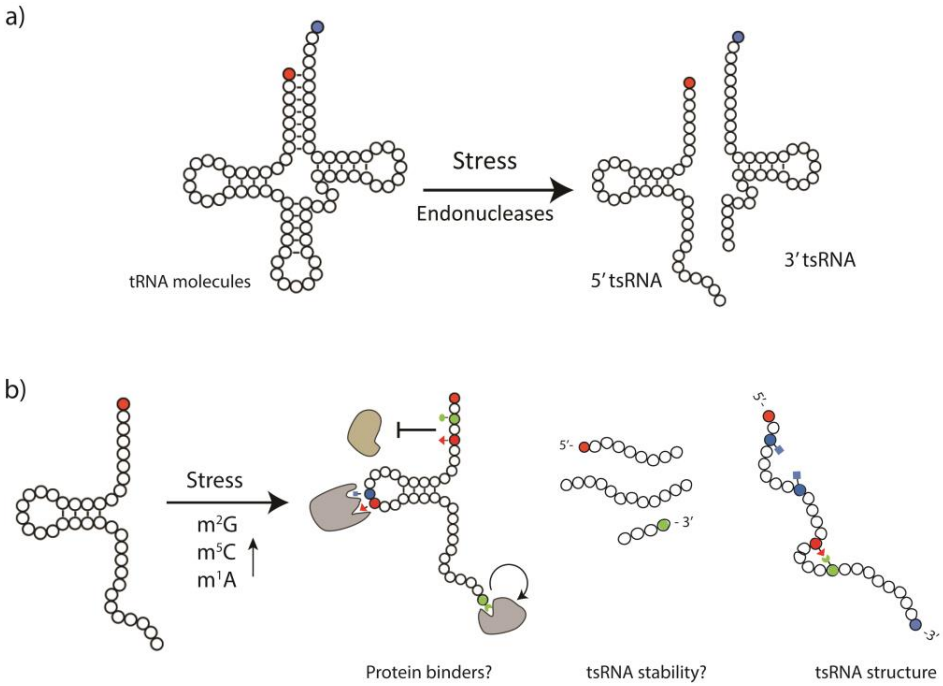

Figure 1: tRNA molecules can be cleaved in the codon loop by specific endonucleases under diverse conditions of stress to produce tsRNA molecules (a). tsRNA modifications change during the stress response. These modifications are thought to impact tsRNA protein binding propensity, convey stability to further processing or guide formation of secondary structures (b).

-Possible mechanisms of tsRNA-mediated transgenerational inheritance

The molecular mechanisms as to how sperm RNAs, in particular specific tsRNAs, induce changes in early embryonic development towards recapitulating paternally acquired traits remain completely unclear. In addition, how initial molecular changes in zygotes are translated into stable information to allow for inter-generational inheritance of acquired traits remains a major puzzle. Under physiological conditions, the amount of sperm RNA delivered into the oocyte is infinitesimally small in comparison to maternally loaded RNAs, suggesting that such paternal "signals" must either act directly on a very few molecules present in the fertilized zygote, or must become amplified to make an impact. Potential mechanisms to achieve this have been proposed (Figure 2):

- *Influence on spatial organization of zygotic proteins and RNAs:* Incoming sperm material could influence the order and polarity of cell divisions as well as the spatial patterning in the developing embryo (Piotrowska et al, 2001). In this regard, small biases in local RNA distribution (through introduction of sperm-derived tsRNAs) could lead to changes in protein and RNA segregation patterns during the first cell division. This could result in asymmetrical gene expression changes in dividing cells, with impact on defining lineage fates in the embryo, thereby influencing the trajectory of further embryonic development (Shi et al, 2015) (Figure 2a).

- *Direct influence on epigenetic regulation of gene expression: S*perm-borne tsRNAs exhibit sequence matches to particular promoters, many of which were derived from transposable element sequences (Chen et al, 2016; Sharma et al, 2016). Indeed, injection of tsRNAs derived from tRNA-Gly-GCC into zygotes repressed expression from genes containing murine endogenous retrovirus (MERVL)-derived promoter sequences (Sharma et al, 2016). These gene expression changes were persistent throughout embryonic development and could even be detected in the pancreatic islets in adult mice indicating the existence of mechanisms that stably propagated gene expression patterns (Chen et al, 2016). RNA-directed DNA methylation is a well-established epigenetic mechanism in plants, and site-specific DNA methylation of transposon sequences by piRNA molecules has been detected in mammals. Whether or not tsRNAs can fulfill similar functions remains to be tested (Figure 2b).

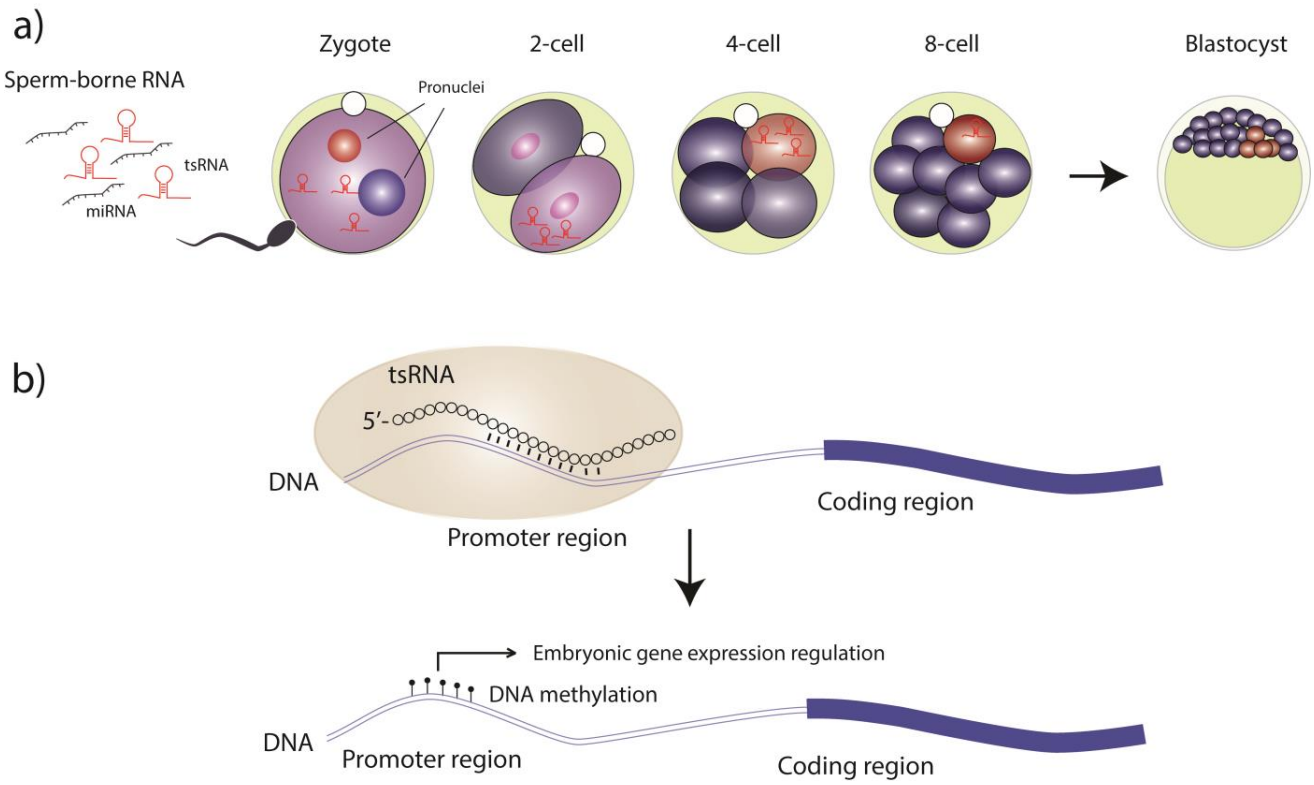

Figure 2: tsRNA molecules introduced by the sperm could act to segregate particular developmentally-important molecules during early embryo development (a). In addition, tsRNA molecules could act to guide deployment of epigenetic marks on their target sequences in the genome (b).

- **Specific Aims**

I have developed a biochemical purification pipeline which enables purifying specific, endogenously produced and fully post-transcriptionally modified tsRNAs. Here, I propose utilizing this methodological workflow to prepare specific tsRNAs that can be manipulated and tested for their activities in early mouse embryos.

More specifically, after injection of specific tsRNAs into in vitro fertilized oocytes I will follow their stability (half-life), subcellular localization and potential effects on EGA during early mouse embryogenesis. These experiments will provide deeper mechanistic insights into the phenomenon of transgenerational inheritance mediated by small non-coding RNAs.

- **Methodology**

To address how exactly tsRNAs affect early development and if post-transcriptional modifications are required for their function I will use a combination of biochemical, single-cell embryo injections and state of the art confocal microscopy.

-Purification of specific endogenous tsRNAs

During my ongoing PhD thesis, a biochemical purification pipeline was established that enables purifying specific, fully modified, endogenous tsRNAs from mammalian cells. tsRNA production can be easily achieved by applying various stress paradigms or through overexpression of endonucleases responsible for tRNA cleavage (i.e. Angiogenin endonuclease). Preliminary experiments showed that this scalable approach allows purifying substantial amounts of endogenous tsRNAs through a combination of HPLC-based separation methods including Ion-Exchange, Affinity and Size Exclusion Chromatography (Figure 3a, 3b). Next-generation-sequencing (NGS) of purified 5' Gly GCC tsRNA showed high specificity of the tsRNA purification output (Figure 3c). Importantly, using highly sensitive LC-MS/MS approach allows determining the modifications status of purified tsRNAs (Figure 3d). Specific RNA modification enzymes have been shown to be important for the process of transgenerational inheritance: namely the (cytosine-5) RNA methyltransferase Dnmt2/Trdmt1 (Zhang et al, 2018; Kiani et al, 2013). To delineate whether 5-methyl cytosine (m^5C) installed by (cytosine-5) RNA methyltransferases have an impact on tsRNA functions in early embryos, specific 5' and 3' tsRNA molecules will be purified from wild type as well as from Dnmt2/Trdmt1$^{-/-}$ mouse embryonic fibroblasts (MEFs). The sequence identity and modification status of endogenously purified tsRNAs will be determined by NGS and LC-MS/MS before their use in zygotic injections. In addition, in-vitro synthesized specific tsRNAs will be used as controls in order to address the contribution of post-transcriptional modifications in tsRNAs.

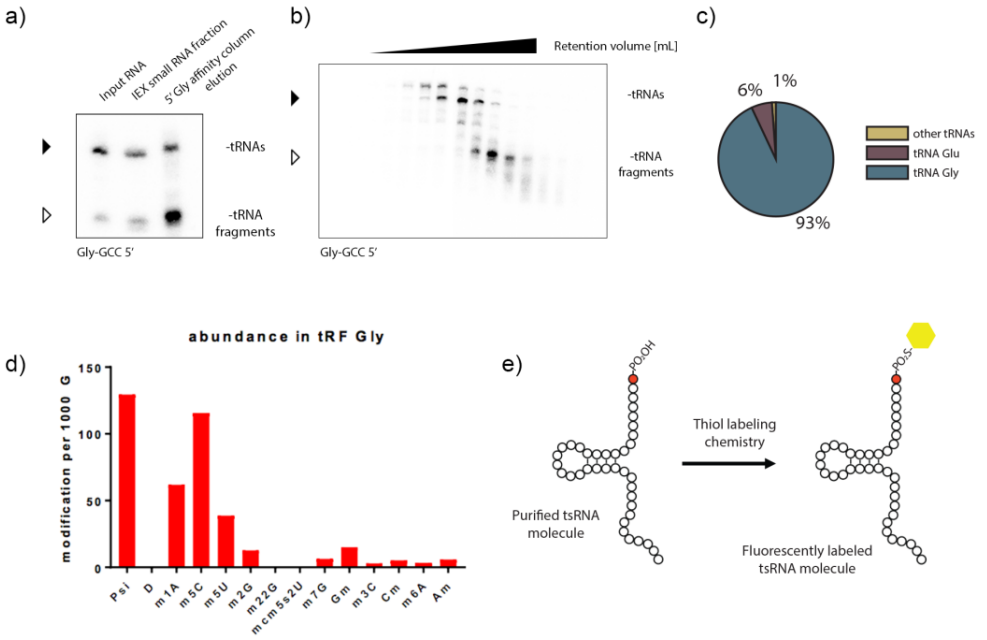

Figure 3: Endogenous tsRNA molecules can be purified by a combination of chromatographic means (a), followed by full length tRNA sepparation by Size Exclusion Chromatography (b). Purification of 5' Gly tsRNA, which was shown to be active in regulating zygotic gene expression (Sharma et al, 2016), shows high specificity in terms of sequence (c) and modifications identity (d). Purified tsRNA molecules will be fluorescently labeled with Alexa 488 fluorophore using thiol specific chemistry (e).

-Labeling of specific tsRNAs

Purified tsRNAs from wild type and (cytosine-5) RNA methyltransferase mutant backgrounds, as well as in vitro synthesized tsRNAs will be fluorescently labeled using thiol specific chemistry (Figure 3e). Firstly, the 5' end of either 5' or 3' tsRNA molecule will be treated with the PNK enzyme in the presence of adenosine 5'-[γ-thio]triphosphate, which results in a 5' thiol group placement. Thiol group, under oxidative environment, can be used to covalently and irreversibly label the purified tsRNA molecule with specific thiol-reactive probes, such as maleimide-conjugated Alexa 488 fluorophore. Alexa fluorophore enables us to follow labeled tsRNA molecules using conventional confocal microscopy during early embryogenesis.

-Microinjections of labelled tsRNAs into mouse zygotes

For the experimental setup I will use an existing microinjection setup at the Department of Cell- and Developmental Biology at the Medical University of Vienna. I will perform injections with the assistance of Dr. Mark Wossidlo, who has extensive experience in manipulating early mammalian embryos. Crucial for the proposed experiments is the reproducible and scalable delivery of tsRNAs into the recipient zygotes without causing damage to the embryo. While the existing set-up is suitable for single cell injections, the reproducibility and successful retrieval of injected zygotes is very inefficient. The piezo-assisted micromanipulation device (Eppendorf PiezoXpert, please find quote attached) is the best state-of-the art available system on the market, which will improve the efficiency of microinjections by reducing cell death and enabling reproducible delivery of RNA cargo, raising the number of viable microinjected embryos for the experimental purposes. This add-on to the existing setup will be used to microinject (fluorophore-tagged) tsRNAs into in vitro fertilized mouse oocytes shortly after fertilization and analyze their role on epigenetic reprogramming and gene expression changes in early embryogenesis. Labeled tsRNA molecules will be injected in the pronuclei, as well as separately in the cytoplasm of the one cell zygotes in order to simulate the influence of the incoming sperm.

-Monitoring subcellular localization of labelled tsRNAs

After injecting fluorescently labelled tsRNAs, I will address the impact of RNA modifications on the stability of injected tsRNAs, comparing the half-life of tsRNAs purified from different genetic sources with synthetic tsRNAs in one-cell, two-cell and later preimplantation embryos cultured in vitro. Next, the subcellular localization of different tsRNA molecules will be determined. More specifically, I will determine the cytoplasmic-nuclear distribution, preference towards the paternal or maternal pronucleus as well as segregation bias towards specialized cells during cell divisions (Figure 2a). To this end, conventional confocal microscopy will be used, in

combination with immuno-labeling of specific markers of paternal/maternal pronuclei. In addition, expression of putative target genes (Table 1) of tsRNAs during EGA at the two-cell stage will be monitored by single embryo RT-qPCR approach. Genes that will be probed are connected to murine endogenous retrovirus (MERVL) elements, which are known to be highly expressed during this stage, and to be important for EGA and the generation of a totipotent two-cell embryo. Further on, co-localization between tsRNA molecules and genomic loci of putative target genes in the zygotic pronuclear genome will be probed by using FISH.

Putative target genes		
Gm4340	Esp24	Arg2
Tdpoz3	Tdpoz4	Cwc22
Dub3	4Tdpoz5	Sp110
Ddr2	Cpb2	Arg2
Bex6	Tcstv3	Cwc22
Apol7b	Gm4567	Sp110

Table 1: Putative candidate genes shown to be affected by tsRNA molecules (Sharma et al, 2016)

In summary, these simple but technically challenging experiments will provide further insight how small non-coding RNA molecules influence early embryo development. Understanding this phenomenon will not only deepen our knowledge how early patterns of gene expression are being set, but also how mechanisms of transgenerational inheritance are set in motion.

- **Wider applications of Eppendorf PiezoXpert**

All experiment proposed rely on precise and reproducible delivery of RNA cargo to the recipient zygote. To this end, I am asking for help for buying state of the art injection apparatus: PiezoXpert (est. 8000 euros). With many genetic experiments based on creating transgenic animals, this device can also be used to inject proteins, DNA and RNA, which can be used for the generation of transgenic mice by genome editing (CRISPR-Cas9) and other interesting tasks in the field of developmental and regenerative biology. Next to microinjection of solutions, this device is also routinely used for the generation of cloned mouse embryos and chimeric embryos by microinjection of somatic nuclei into enucleated oocytes and pluripotent stem cells into blastocysts. The acquisition of this device would therefore be also enable other groups in Vienna in the field to pursue interesting questions in early development and stem cell biology.

- **References**

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