



# The impact of environmental insult on mouse epididymal spermatozoa

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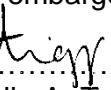
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I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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### ACKNOWLEDGMENT OF AUTHORSHIP

I hereby certify that the work embodied in this thesis contains published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written declaration endorsed in writing by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

By signing below, I confirm that Natalie Trigg contributed upward of 50% towards data collection/analysis and manuscript preparation for all the papers / publications included in this thesis

Signed:   
Brett Nixon

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*This thesis was written and researched on Awabakal Lands.*

*Wherever we walk in Australia, we walk on Aboriginal land.*

## PUBLICATIONS AND AWARDS ARISING FROM THIS THESIS:

### Publications:

#### Chapter 1: Introduction and Literature Review

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## STATEMENT OF CONTRIBUTION

I attest that the Research Higher Degree candidate Natalie Trigg has contributed upward of 50% towards data collection/analysis and manuscript preparation for all the publications included in this thesis for which I am co-author.

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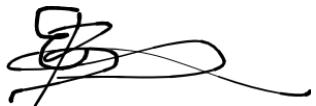
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## CONFERENCE PROCEEDINGS RELEVANT TO THESIS:

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1. **Trigg, N.A.**, Skerrett-Byrne, D.A., Xavier, M.J., Zhou, W., Anderson, A.L., Stanger, S.J., De Iuliis, G.N., Roman, S.D., Eamens, A.L., Nixon, B (2020) Acrylamide exposure drives alterations to the small RNA landscape of mature spermatozoa and influences early embryo gene expression. Society for Reproductive Biology Virtual Awards. *Oral Presentation*

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2. **Trigg, N.A.**, Roman, S.D., Eamens, A.L., Xavier, M.J., Nixon, B (2019) The impact of acute acrylamide exposure on the small RNA profile of spermatozoa. 24<sup>th</sup> Annual Newcastle University Higher Degree Research Conference, Newcastle, Australia. *Oral Presentation*
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### 2018:

5. **Trigg, N.A.**, Roman, S.D., Eamens, A.L., Xavier, M.J., Nixon, B. (2018) The impact of acute acrylamide exposure on the small RNA profile of spermatozoa. 23<sup>rd</sup> Annual Newcastle University Higher Degree Research Conference. Newcastle, Australia. *Poster Presentation*
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8. **Trigg, N.A.**, De Iuliis, G.N., Bromfield, E.G., Eamens, A.L., Nixon, B. (2018). The impact of environmental insults on the small non-protein-coding RNA profile of spermatozoa. Australian Society for Medical Research (ASMR) Satellite Scientific Meeting. Newcastle, Australia. *Oral Presentation*

### 2017:

9. **Trigg, N.A.**, De Iuliis, G.N., Bromfield, E.G., Eamens, A.L., Nixon, B. (2017) Regulation of the sperm epigenome by epididymosomes: A new paradigm. 22<sup>nd</sup> Annual Newcastle University Higher Degree Research Conference. Newcastle, Australia. *Oral Presentation*

## ADDITIONAL PUBLICATIONS:

### 2020

1. Fraser, B.A., Miller, K., **Trigg, N.A.**, Smith, N.D., Western, P.S., Nixon, B., and Aitken, J.R. (2020) A novel approach to non-surgical sterilization; application of menadione-modified gonocyte-targeting M13 bacteriophage for germ cell ablation in utero. **Pharmacology Research & Perspectives** <https://doi.org/10.1002/prp2.654>
2. Tamessar. C.T., **Trigg. N.A.**, Sharkey. D.J., Robertson S.A., Bromfield. E.G., Nixon. B., and Schjenken. J.E. (2020) Roles of male reproductive tract extracellular vesicles in reproduction. **American Journal of Reproductive Immunology**, <https://doi.org/10.1111/aji.13338>

### 2019

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2. Nixon, B., De Iuliis, G. N., Dun, M. D., Zhou, W., **Trigg, N. A.**, and Eamens, A. L. (2019), Profiling of epididymal small non-protein-coding RNAs. **Andrology**. <https://doi:10.1111/andr.12640>

### 2018

1. Houston, B.J., Nixon, B., Martin, J.H., De Iuliis, G.N., **Trigg, N.A.**, Bromfield, E.G., McEwan, K.E., and Aitken, R.J. (2018) Heat exposure induces oxidative stress and DNA damage in the male germ line. **Biology of Reproduction** <https://doi.org/10.1093/biolre/ioy009>

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**Faculty 1000 recommendation** for review entitled 'The contribution of epididymosomes to the sperm small RNA profile' (2019)

**Best PhD Student Poster Award | Newcastle University, School of Environmental and Life Sciences 23<sup>rd</sup> annual HDR conference | Awarded by the University of Newcastle (2018)**

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## **ABBREVIATIONS:**

AA-E – acrylamide exposure timed to collect mature cauda spermatozoa exposed as epididymal sperm

AA-S – acrylamide exposure timed to collect mature cauda spermatozoa exposed as spermatocytes

ART – assisted reproductive technologies

EV – extracellular vesicles

ES – epididymosomes (also referred to as epididymal extracellular vesicles)

ICSI – intracytoplasmic sperm injection

IPA® – ingenuity pathway analysis

IVF – *in vitro* fertilisation

mECap18 – SV40-immortalised mouse caput epididymal epithelial cell line 18

miRNA – microRNA

piRNA – piwi-interacting RNA

sRNA / sncRNA – small non-protein coding RNA

TF – transcription factor

tRF – transfer RNA derived RNA fragments

## ABSTRACT:

The possibility of inheritance that deviates from canonical Mendelian rules (epigenetic / non-genetic inheritance) has been considered since the beginning of genetics research, however, only in recent years has experimental work provided the evidence needed to substantiate epigenetic inheritance. Indeed, models of epigenetic inheritance have now been established in species ranging from worms to mice. The putative ‘information carriers’ responsible for epigenetic inheritance, include DNA and histone modifications, chromatin modifications and, small non-coding RNAs (sncRNAs) and the modifications they harbour. Recently, several lines of evidence have supported sperm-borne sncRNA as an integral intergenerational signalling molecule following their delivery to the oocyte at the moment of fertilisation. It is also now apparent that the sperm sncRNA profile is dynamic and susceptible to modification during sperm maturation and in response to paternal environmental and lifestyle factors; each of which have potentially significant post-fertilisation consequences. While, the importance of the sperm sncRNA profile emerges, what remains less clear are the molecular mechanism(s) underpinning the response to environmental insult that leads to an altered sperm sncRNA profile. The studies in this thesis were designed to begin to bridge this important knowledge gap. Specifically, we aimed to investigate how paternal exposure to environmental factors influence the sperm sncRNA profile and the consequences of the delivery of an altered sncRNA profile to the oocyte (Chapter 2).

The sncRNA profile of spermatozoa undergoes major remodelling during the sperm cells passage of the epididymis. Moreover, the balance of evidence has implicated the epididymis as a vulnerable site in which sperm acquire environmental signals, such as an altered sncRNA profile. Owing to the transcriptionally and translationally quiescent state of epididymal spermatozoa, modification to the sncRNA profile is facilitated by the complex luminal microenvironment. A key component of which are small membrane bound vesicles, termed epididymosomes. These vesicles are produced by the epididymal epithelial cells and deliver a diverse cargo of macromolecules, including sncRNA, to spermatozoa. Additionally, epididymosomes have recently been implicated in delivery of an altered sncRNA profile to sperm under paternal stress conditions. Hence, in examining the mechanism(s) driving sperm sncRNA changes, we focused on epididymosomes as mediators of this dialogue and more specifically, on the cognate receptor-ligand(s) that underpin epididymosome-sperm adhesion (Chapter 3).

The data presented within this thesis confirms the acute sensitivity of the sperm sncRNA payload to the environmental toxicant, acrylamide, encountered during their post testicular development. We have traced the differential accumulation of miRNAs to coincide with sperm transit of the proximal (caput) epididymal segment. Indeed, we identified alterations in the epididymosomes secreted by the caput epididymis following environmental insult. In expanding this mechanistic investigation, we profiled the proteome of the caput epididymal epithelium and

revealed that acrylamide exposure alters the expression of a subset of proteins. In resolving a causal link, we identified the increased expression of seven transcription factors in the caput epithelium of acrylamide exposed mice, each of which have been implicated in the regulation of acrylamide-sensitive miRNAs. In identifying the consequences of an altered sperm sncRNA profile following acrylamide exposure our analysis revealed a subset of dysregulated genes in embryos fertilised by exposed sperm. This gene dysregulation was demonstrated to be in part driven by the sncRNA changes in the sperm and thus substantiate sperm-borne sncRNAs as important epigenetic messengers.

Having confirmed an integral role for epididymosome mediated communication in facilitating a response to paternal environmental exposure (acrylamide), we next sought to further our understanding of the mechanistic basis underlying the interaction between epididymosomes and recipient sperm. In exploring the proteomic composition of epididymosomes, previous work from our laboratory identified a putative role for an epididymosome resident ligand, milk fat globule-EGF factor 8 (MFGE8), in the efficient transfer of cargo from epididymosomes to spermatozoa. MFGE8 has been implicated in the adhesion of extracellular vesicles (EVs) isolated from non-reproductive tissues to recipient cells and this finding indicated a conserved function for MFGE8 in EVs isolated from the epididymis. Therefore, the remaining studies herein focused on the role of MFGE8 as a key molecular ligand in this intercellular form of communication in the epididymis (Chapter 3). We utilised an immortalised caput epididymal (mECap18) cell line as a model and first confirmed its suitability with which to study the mechanism(s) of sperm-epididymosome interaction. Through additional inhibition studies and the ablation of MFGE8 functional domains, we identified MFGE8 as being of fundamental importance for efficient sperm-EV interaction. However, the failure to completely block sperm-EV interaction indicates redundancy in the EV tethering mechanisms and highlights the need for further research to gain a complete understanding of the cognate receptors and ligands that mediate this interaction.

Taken together, the findings of this thesis contribute to our understanding of the sncRNA profile of mature mouse spermatozoa and its dynamic response to environmental insult. Importantly, these studies have advanced our knowledge of the molecular basis of epididymosome-sperm interactions, the importance of this intercellular communication in directing sperm sncRNA changes following stress, the mechanistic understanding of how paternal exposures affect remodelling of the sperm small RNA profile and the consequences of such changes for embryonic development.

## CHAPTER ONE: INTRODUCTION & LITERATURE REVIEW

# The contribution of epididymosomes to the sperm sRNA profile



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## CHAPTER OVERVIEW:

The sperm sncRNA profile is a multi-layered and versatile RNA code that is dynamically modified during epididymal transit and displays considerable plasticity in response to paternal exposure to a range of lifestyle and environmental factors. Furthermore, sperm-borne sncRNA are now recognised as a source of paternal hereditary information. In this literature review, we document the diverse profile of sncRNAs that spermatozoa harbour and discuss the dynamic reshaping that occurs to the sncRNA landscape as the sperm cell traverses the epididymis. We pay particular attention to the role of epididymosome-sperm communication in contributing to the establishment of the sperm RNA profile during their epididymal transit. At current, epididymosome mediated delivery has been proposed as the putative driver underpinning the maturation of the sperm sncRNA profile during epididymal transit and recently implicated in orchestrating a sperm sncRNA response to environmental insult. We surveyed the relevant literature and generated a comprehensive list of sncRNAs that experience altered accumulation across multiple stress models and highlight recent studies providing supportive evidence for the role of epididymosomes in facilitating these changes. Such alterations to the sperm sncRNA landscape have been increasingly linked to significant post-fertilisation consequences extending across multiple generations. Overall, the collective evidence reviewed herein demonstrates the importance of epididymosome-mediated transport of sncRNA to spermatozoa in establishing the sperm epigenome; an important contributor to embryonic development and subsequent offspring health. Finally, we identify the remaining unanswered questions in the field and the need to continue research to examine the mechanism(s) by which paternal exposure leads to a change in the sperm sncRNA profile and discern the consequences of delivering an altered sperm sncRNA profile to the embryo.

## The contribution of epididymosomes to the sperm small RNA profile

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### Abstract

It is now well established that mature spermatozoa harbour a rich and diverse profile of small non-protein-coding regulatory RNAs (sRNAs). There is also growing appreciation that this sRNA profile displays considerable plasticity, being altered in response to paternal exposure to a variety of environmental stressors. Coupled with evidence that upon delivery to the oocyte at the moment of fertilisation, sperm-borne sRNAs are able to influence both early embryonic development and the subsequent health of the offspring, there is now interest in both the timing and degree of change in the composition of the sRNA cargo of sperm. Models in which such epigenetic changes are linked to the spermatogenic cycle are seemingly incompatible with the lack of overt phenotypic changes in the spermatozoa of affected males. Rather, there is mounting consensus that such changes are imposed on sperm during their transit and storage within the epididymis, a protracted developmental window that takes place over several weeks. Notably, since spermatozoa are rendered transcriptionally and translationally silent during their development in the testes, it is most likely that the epididymis-documented alterations to the sperm sRNA profile are driven extrinsically, with a leading candidate being epididymosomes: small membrane enclosed extracellular vesicles that encapsulate a complex macromolecular cargo of proteins and RNAs, including the sRNAs. Here, we review the role of epididymosome–sperm communication in contributing to the establishment of the sperm sRNA profile during their epididymal transit.

*Reproduction* (2019) 157 R209–R223

### Introduction

The volume of studies reporting the inheritance of non-genome-derived information have increased over the past decade. This so-called epigenetic mode of inheritance challenges long held paradigms of classic Mendelian genetics by offering a rational mechanism for the transmission of environmental information to the offspring, which can conceivably lead to novel phenotypic outcomes. Mounting evidence for the existence of such alternate routes of inheritance includes examples from epidemiological analysis of humans and studies performed in model species ranging from worms to mammals (Kaati *et al.* 2007, Dunn & Bale 2009, Lim & Brunet 2013). Collectively, these studies provide an important precedence for the potential transmission of paternal traits acquired by environmental conditions to the offspring, suggesting the existence of additional ‘information carriers’ beyond that of an individual’s genome. Among the information carriers potentially responsible for relaying non-genetic information between generations, considerable attention has focused on small non-protein-coding regulatory RNAs (sRNAs). Accordingly, mature spermatozoa harbour

a rich and diverse sRNA profile, the foundations of which are established during the testicular phases of their development. Notably, however, recent evidence has shown that the sperm sRNA profile displays considerable plasticity, being substantially remodelled as the cells traverse the epididymis, an integral part of the extra-testicular male reproductive tract (Nixon *et al.* 2015). Importantly, alteration of the sperm sRNA profile during epididymal transit has recently been shown to be essential for the production of functionally mature spermatozoa capable of supporting not only fertilisation, but also normal embryonic development (Conine *et al.* 2018). Moreover, the sRNA profile of sperm has also been demonstrated to be altered in response to a male’s exposure to a number of environmental insults, with such alterations having been linked with significant post-fertilisation consequences, commonly converging on the transmission of altered behavioural and metabolic phenotypes to the offspring (Fullston *et al.* 2013, Rodgers *et al.* 2013, Short *et al.* 2016). The demonstration that such phenotypic changes can be recapitulated via the direct microinjection of sRNAs harvested from the sperm of insult-exposed males into otherwise ‘normal’ fertilised oocytes, effectively

eliminates the contribution of germ-line-independent paternal factors, and further, implicates sRNAs as the causative agents (Gapp *et al.* 2014, Grandjean *et al.* 2015, Rodgers *et al.* 2015, Chen *et al.* 2016a). Nevertheless, while the list of environmental insults that influence the composition of the sRNA cargo of sperm continues to grow, the mechanism(s) responsible for mediating these changes to the sperm epigenetic landscape remains uncertain. Among the plausible scenarios, there is mounting consensus that such changes are imposed on sperm during their transit and storage within the epididymis. Notably, since spermatozoa are rendered transcriptionally and translationally silent before departing the testes (Kierszenbaum & Tres 1975, Steger 1999), it is most likely that any alterations to the sperm sRNA profile in the epididymis are driven extrinsically, with a leading candidate being epididymosomes: small membrane enclosed extracellular vesicles that encapsulate a complex macromolecular cargo of RNA and protein. Indeed, there is recent evidence that chronic exposure of a male to insults such as alcohol elicits a change in their epididymosome sRNA cargo, a change that closely parallels the altered sRNA profile of exposed spermatozoa (Rompala *et al.* 2018). Given the hypothesized contribution of the sperm sRNA profile to the offspring, important emphasis should be placed on documenting the contribution of epididymosome-mediated communication with the sperm as these cells transit the epididymis. Hence, we discuss here the role of epididymosomes in modulating the sperm sRNA profile during epididymal transit, paying particular attention to their contribution to the sRNA profile of sperm, a profile that is altered by paternal exposure to stress. Further, we also discuss the relevance of an altered sperm sRNA profile on the subsequent development of the embryo and the health of offspring.

### The action of small RNA species documented in spermatozoa

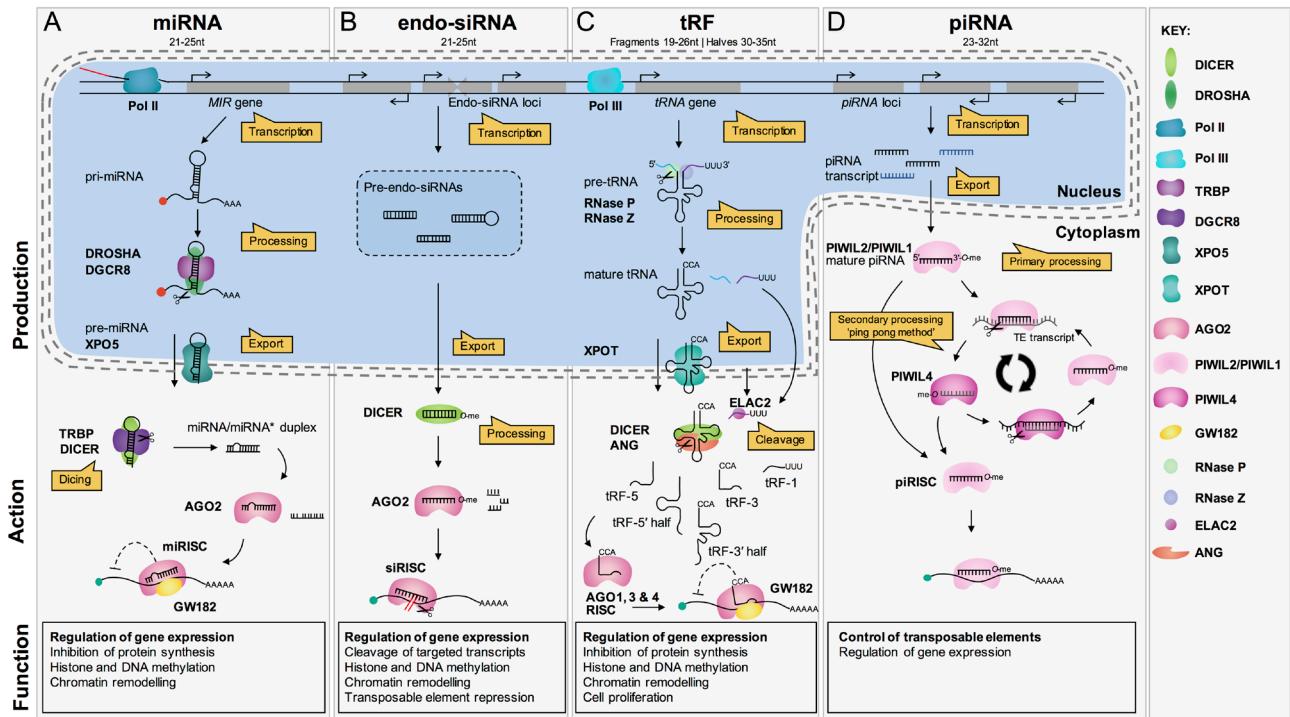
It is becoming increasingly accepted that spermatozoa are more than mere vectors for conveying the paternal haploid genome to the oocyte. Indeed, beyond the paternal genome, spermatozoa harbour non-genetic information that can be propagated to the ensuing embryo. This epigenetic information includes a diverse RNA population (Chen *et al.* 2016b), and from this global RNA pool, sRNAs command considerable attention.

In spermatozoa harvested from the proximal caput segment of the epididymis, miRNAs are the most abundant sRNA species (Hutcheon *et al.* 2017), accounting for approximately half of the global sRNA population. miRNA production begins with the transcription and folding of the primary-miRNA (pri-miRNA) transcript (Ambros *et al.* 2003). Next, the pri-miRNA is processed to yield a mature miRNA duplex via the action of two

RNase III-like endonucleases, DROSHA and DICER, and their partnering double-stranded RNA (dsRNA)-binding (DRB) proteins, DGCR8 (DiGeorge syndrome critical region 8) and TRBP (transactivation-responsive RNA-binding protein) (Fig. 1A) (Lee *et al.* 2003, Haase *et al.* 2005, Ha & Kim 2014). The miRNA/miRNA\* duplex is subsequently loaded into the endonuclease, ARGONAUTE 2 (AGO2). While either strand of the miRNA/miRNA\* duplex has the potential to act as a functional sRNA, only one strand is retained by AGO2 to form an activated miRNA-induced silencing complex, miRISC (Hu *et al.* 2009, O'Brien *et al.* 2018). miRNAs direct miRISC to regulate target gene expression by binding to lowly complementary target sequences, and in mammals, miRNA target sequences are almost exclusively harboured in the 3' untranslated region (3' UTR) of the targeted transcript (Lee *et al.* 1993, Bartel 2009). Once miRISC is bound to the 3' UTR of the targeted transcript, miRISC interferes with gene expression via blocking translation (Ha & Kim 2014) (Fig. 1A).

Another developmentally important sRNA species harboured by spermatozoa are the endogenous siRNAs (endo-siRNAs) (Song *et al.* 2011). Double-stranded RNA transcripts of perfect complementarity serve as precursor substrates for endo-siRNA production. These substrates are exported to the cytoplasm where they are processed by DICER to produce a mature endo-siRNA (Song *et al.* 2011). Once liberated from its precursor, the mature endo-siRNA is loaded into AGO2 to form an active siRISC to target mRNAs that harbour highly complementary sequences for endonucleolytic cleavage (Czech *et al.* 2008, Kawamura *et al.* 2008) (Fig. 1B). Research performed to date in plants, yeast and more recently flies, has revealed that this class of regulatory sRNA directs an additional function as an epigenetic regulator, via either RNA-directed DNA methylation (RdDM), or chromatin modification (Fagegaltier *et al.* 2009, Verdel *et al.* 2009). However, whether spermatozoa harboured endo-siRNAs are involved in an analogous pathway in animals remains to be determined. Nevertheless, sperm-borne endo-siRNA-directed gene expression regulation has been linked to critical early embryo development events in mice (Yuan *et al.* 2016).

Interestingly, distinct to the predominance of the miRNA class of sRNA in caput sperm, the most abundant sRNA class harboured by mature cauda sperm, are the tRFs, or tRNA-derived fragments (Sharma *et al.* 2016, Hutcheon *et al.* 2017). Transcription and processing of a precursor tRNA (pre-tRNA) transcript occurs within the nucleus to produce a mature tRNA (Fig. 1C). Once exported to the cytoplasm, the tRNA is able to fulfil its primary function in protein translation on the ribosomes. However, beyond their fundamental role as a linker molecule in translation, the specific fragmentation of mature tRNA transcripts by the endonucleases angiogenin (ANG) and DICER to produce tRFs, affords



**Figure 1** The production and function of small RNA species harboured by mouse spermatozoa. Mouse spermatozoa harbour a diverse repertoire of sRNAs, including miRNA, endo-siRNA, tRF and piRNA classes (Hutcheon *et al.* 2017). The individual steps involved in the production, and subsequent action, of each class of sRNA that accumulates in mouse spermatozoa is distinct. (A) *miRNA*: MIR genes are transcribed by RNA polymerase (Pol) II to form a primary-miRNA (pri-miRNA) and the pri-miRNA is processed by DROSHA and DGCR8 to generate the precursor miRNA (pre-miRNA) (Lee *et al.* 2003). Post pre-miRNA nuclear export by exportin-5 (XPO5) (Yi *et al.* 2003), the pre-miRNA is processed by DICER and TRBP to generate the miRNA/miRNA\* duplex (Haase *et al.* 2005), and either duplex strand is loaded into ARGONAUTE2 (AGO2) to form miRISC. Together with protein, GW182, miRISC represses miRNA target transcript translation (reviewed in Ha & Kim 2014). (B) *Endo-siRNA*: perfectly dsRNA molecules serve as endo-siRNA precursors (pre-endo-siRNA), and post nuclear export, the pre-endo-siRNA is processed by DICER to produce a siRNA/siRNA\* duplex. Either duplex strand is loaded by AGO2 to form siRISC, and siRISC regulates siRNA target gene expression via AGO2-catalysed mRNA cleavage (Song *et al.* 2011). (C) *tRF*: precursor tRNAs (pre-tRNA) are transcribed from tRNA genes by RNA Pol III, and are subsequently processed by RNase P and RNase Z prior to nuclear export by exportin-T (XPOT). Mature tRFs are generated directly by the action of angiogenin (ANG), or via further DICER processing (Cole *et al.* 2009, Fu *et al.* 2009). The resulting tRF is loaded into AGO1, AGO3 or AGO4 to direct target gene translational repression. (D) *piRNA*: intergenic region-derived transcripts are loaded into PIWI proteins based on their 5' terminal nucleotide (Ishizu *et al.* 2012), and subsequently undergo 3' to 5' exonucleolytic trimming and 2'-O-methylation, to form mature primary piRNAs (Kirino & Mourelatos 2007). The primary piRNA is then either; (1) loaded directly into RISC to target transcripts for cleavage-mediated silencing, or; (2) used by other PIWI proteins (PIWIL2/PIWIL1) to direct cleavage-mediated silencing of transposable element (TE)-derived transcripts in the secondary piRNA production pathway, the piRNA ‘ping-pong’ pathway (Iwasaki *et al.* 2015).

a new regulatory role to these once thought static contributors to gene expression (Cole *et al.* 2009, Fu *et al.* 2009, Lee *et al.* 2009). Post production, mature tRFs are loaded into AGO-catalysed RISC and thereafter direct either transcriptional or translational repression, by targeting the promoter region, or the 5' UTR, of a targeted gene/target gene transcript. Such activity has, in turn, been linked to either transcriptional or translational repression of maternal transcripts in the embryo post fertilisation (Chen *et al.* 2016a, Schuster *et al.* 2016a).

Genomic integrity is of particular importance in the gamete destined to form a new organism. In terms of transposon or retrotransposon activity, the genome is afforded protection by an additional species of sRNA, the piRNAs. The piRNA production pathway occurs via a DICER-independent mechanism. Namely, nascent

single-stranded RNAs transcribed from intergenic regions of the genome are loaded into PIWI (P-element induced wimpy testes) proteins (Ishizu *et al.* 2012), with the PIWI-loaded transcript subsequently undergoing 3' to 5' exonucleolytic trimming and 2'-O-methylation at the 3' terminus to form a mature, primary piRNA (Kirino & Mourelatos 2007). Primary piRNAs can on occasion, trigger the generation of secondary piRNA sRNAs via a production pathway known as the ping-pong cycle (Iwasaki *et al.* 2015) (Fig. 1D). The main function of piRNAs to mediate suppression of retrotransposon activity is particularly important in the context of spermatogenesis and is also of central relevance during early embryogenesis (Carmell *et al.* 2007). However, the role of piRNAs extends beyond this canonical regulatory function to gene expression regulation via modulation

of the epigenetic status of protein-coding genes (Aravin *et al.* 2008, Thomson & Lin 2009).

### Spermatozoa harbour a diverse small RNA profile that is potentially delivered to the oocyte at fertilisation

Due to the transcriptionally and translationally inert state of spermatozoa, the RNA transcripts identified in this cell type were originally thought to be inconsequential remnants of untranslated mRNA stores generated during spermatogenesis. However, it is now understood that sperm sRNAs actively contribute to sperm function, early embryo development and transgenerational epigenetic inheritance. Therefore, sRNAs may potentially hold diagnostic value for assessment of the fertility status of a male (Salas-Huetos *et al.* 2016). This potential has served as a catalyst for several recent studies that utilised either a microarray or high-throughput sequencing approach to catalogue the RNA profile of spermatozoa. Such an approach not only confirmed a complex protein-coding RNA profile for sperm (i.e. mRNA transcripts; Miller & Ostermeier 2006), but additionally documented numerous species of non-protein-coding RNA, including numerous classes of sRNA (Ostermeier *et al.* 2005, Krawetz *et al.* 2011). Indeed, as documented earlier, the sRNAs that accumulate in mature spermatozoa include an abundance of miRNAs, endo-siRNAs, tRFs and piRNAs, as well as numerous additional sRNA molecules derived from the snRNA, snoRNA and rRNA molecules (Krawetz *et al.* 2011, Song *et al.* 2011, Peng *et al.* 2012). In recognition of increased interest in sperm-borne RNAs, a comprehensive database featuring expression data for both the large (>200 nt) and small (<200 nt) RNAs identified in the spermatozoa of several model species has been curated (SpermBase; Schuster *et al.* 2016b). The searchable SpermBase repository contains data for mouse, rat, rabbit and human spermatozoa, making it a particularly valuable resource for assessment of cross-species conservation of sperm RNA profiles.

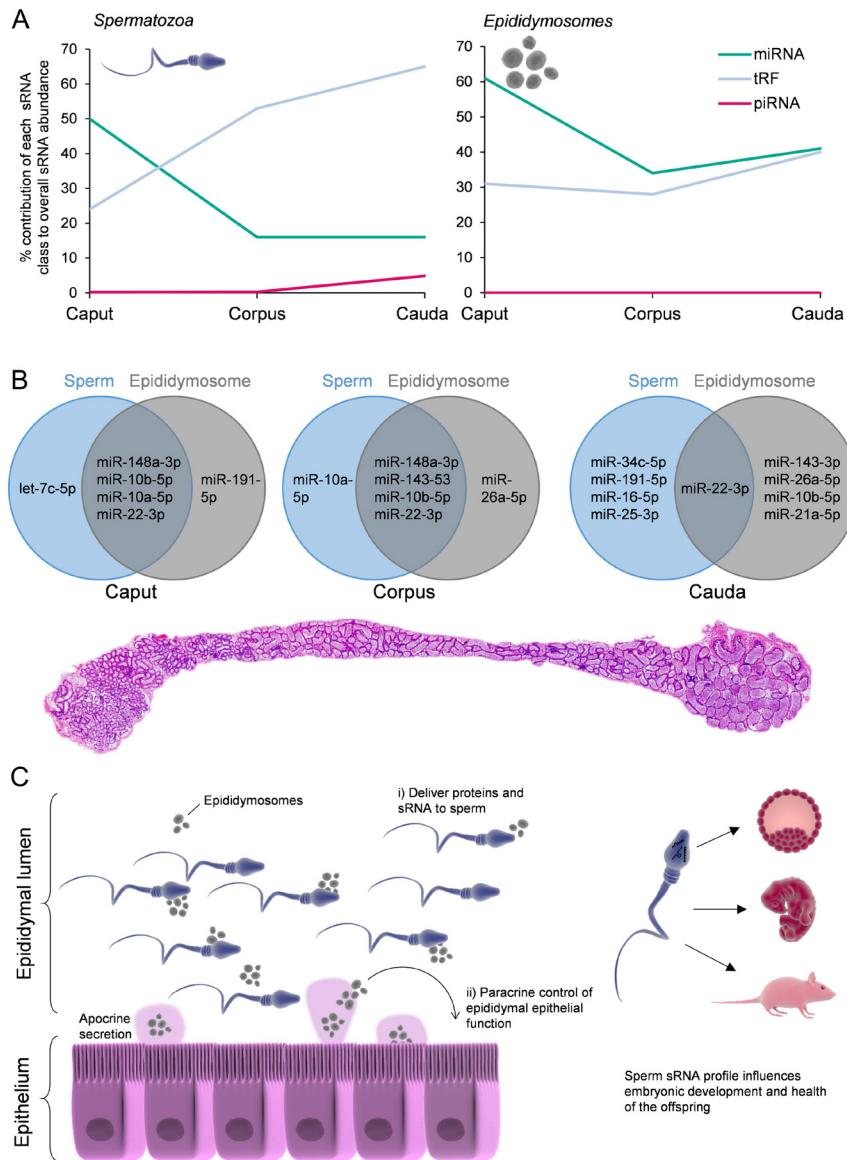
While the biological significance of each of the thousands of distinct sperm-borne RNA species delivered to the oocyte remains to be determined, there is nonetheless growing evidence that highlights the importance of sRNAs in influencing the trajectory of early embryo development and the subsequent later life health of offspring (Chen *et al.* 2016b, Guo *et al.* 2017). One notable example is miRNA, *miR-34c*, which ranks amongst the most abundant miRNAs in human spermatozoa, and a miRNA that is also present in mouse spermatozoa and zygotes, but not in unfertilised female gametes in either of these species (Liu *et al.* 2012). Detailed analysis of *miR-34c* revealed that this miRNA may direct an essential role in regulating the first embryonic cleavage event following fertilisation (Liu *et al.* 2012). However, subsequent studies have shown

that the sperm of mice harbouring a knockout mutation in the *miR-34c* encoding sequence retain their ability to fertilise oocytes and support normal embryonic development, thus casting doubt on the 'actual' biological role of this miRNA in both sperm development and oocyte fertilisation (Yuan *et al.* 2015). Subsequent studies have expanded on this theme, utilising strategies such as the germline-specific ablation of the DICER-encoding gene, the endonuclease responsible for miRNA, endo-siRNA and tRF production (Song & Rossi 2017). This conditional knockout mouse model produced spermatozoa partially deficient in miRNA and endo-siRNA production, and which additionally failed to support normal preimplantation embryonic development (Yuan *et al.* 2016). However, the same study demonstrated that this detrimental phenotype could be rescued via microinjecting fertilised oocytes with the total RNA pool extracted from the spermatozoa of wild-type males (Yuan *et al.* 2016).

### The small RNA profile of spermatozoa is markedly altered during epididymal transit

Spermatozoa leave the testis harbouring a defined sRNA profile consisting of thousands of sequences mapping to all the major sRNA classes (Jodar *et al.* 2013, Sharma *et al.* 2016). Notably however, the sRNA landscape of spermatozoa is by no means static. Instead, the sperm sRNA profile undergoes considerable remodelling during the post-testicular maturation of these cells within the epididymis (Fig. 2A). In tracking the spatiotemporal distribution of the major classes of sperm sRNA, it has been shown that a proportion of sRNAs are maintained at their post-testicular levels as sperm transit the epididymis, while the composition of the remainder of the sRNA pool is altered (Nixon *et al.* 2015, Sharma *et al.* 2016). Illustrative of this remodelling, miRNAs account for approximately half of the global sRNA population of immature spermatozoa that are delivered into the caput epididymis. By comparison, the contribution of miRNAs amounts to only ~16% of the global sRNA population of mature spermatozoa isolated from the distal cauda segment of the epididymis. Coincident with the reduction in miRNA abundance, tRF accumulation returns a reciprocal abundance trend, with the tRF class accounting for 65% of the total sRNA population of cauda sperm: equating to an almost three-fold elevation in abundance compared to caput spermatozoa (24%) (Peng *et al.* 2012, Sharma *et al.* 2016, Hutcheon *et al.* 2017). Further, the abundance of each of the other sRNA classes is also altered during epididymal transit, with notable decreases in abundance to the snoRNA, snRNA and rRNA sRNA classes, and a substantial enrichment to the piRNA sRNA class in cauda sperm (Hutcheon *et al.* 2017) (Fig. 2A).

Remodelling of the sperm sRNA profile during their epididymal transit has recently been linked to the



**Figure 2** Epididymosomes modulate the sperm epigenome during epididymal transit. (A) The proportion of the three prominent small RNA (sRNA) classes implicated in transgenerational inheritance, microRNAs (miRNA), transfer RNA-derived fragments (tRFs) and PIWI interacting RNAs (piRNA). The line graphs depict the percentage of each of the sRNA classes contributing to the overall sRNA population in sperm (left) and epididymosomes (right) sampled from the caput, corpus and cauda mouse epididymis. (B) Venn diagrams illustrating the five most abundant miRNAs in sperm and epididymosomes sampled from the caput, corpus, and cauda segments of adult mouse epididymis. (C) Epididymosomes are released from the epididymal epithelium by apocrine secretion into the epididymal lumen. In the lumen, epididymosomes are able to, (i) interact with transiting spermatozoa and deliver cargo, including sRNA and (ii) effect a form of paracrine regulation by interacting with neighbouring epithelial cells. Epididymosomes play a role in modulating the sRNA profile of sperm, as they transit the epididymis. The population of sperm sRNA is subsequently delivered to the oocyte upon fertilisation and influences embryonic development and the health of the offspring. Figure adapted from Nixon *et al.* (2015), Reilly *et al.* (2016), Hutcheon *et al.* (2017) and Zhou *et al.* (2018).

functional transformation of the sperm cell (Conine *et al.* 2018). Specifically, embryos generated using intracytoplasmic sperm injection (ICSI) of immature caput sperm displayed signatures of aberrant pre-implantation signalling and, as a consequence, consistently failed to implant. Notably, no such defects were observed in embryos generated using either cauda epididymal spermatozoa or those sampled from the

testis. Furthermore, and of considerable interest, was the authors demonstration that these phenotypic aberrations were ameliorated via the co-injection of embryos with the sRNA cargo extracted from cauda epididymosomes (Conine *et al.* 2018). In contrast to this recent finding however, are the previous studies that reported that sperm isolated from the caput epididymis of mice (Suganuma *et al.* 2005), as well as sperm harvested from the human

testis (Greco *et al.* 2005, Weissman *et al.* 2008), are capable of supporting fertilisation and embryonic development following ICSI, despite harbouring contrasting sRNA profiles (Hutcheon *et al.* 2017, Sharma *et al.* 2018). At present, the reason(s) for these apparently contradictory results remain to be determined.

Whilst we clearly still have much to learn about the physiological importance of the reshaping of the sperm sRNA profile as these cells traverse the epididymis, we also remain unsure of the precise mechanisms responsible for driving these alterations. One plausible explanation for the apparent reduction in individual miRNAs, and potentially other classes of sRNAs, is that these transcripts are packaged within the cytoplasmic droplet (Nixon *et al.* 2015); a remnant of the germ cell cytoplasm, which remains adhered to the neck region of testicular spermatozoa, but thereafter, gradually migrates distally along the tail before being shed from mature spermatozoa. Thus, by the time murine spermatozoa reach the cauda epididymis, only approximately half the population still possess cytoplasmic droplets and their encapsulated cargo. More enigmatic however, are the mechanism(s) responsible for sRNA acquisition during epididymal transit, particularly given that spermatozoa are transcriptionally quiescent and therefore incapable of the *de novo* production of sRNA precursor molecules. Spermatozoa must therefore rely on an alternate extrinsic mechanism, with potentially important contributions being made by the epididymal luminal environment in which the sperm are bathed. One possible route for sperm sRNA acquisition is delivery via RNA-binding proteins (RBPs): a sRNA transport route documented in other tissues (Wang *et al.* 2010, Arroyo *et al.* 2011). Indeed, the RBP class of protein have previously been assigned numerous roles in spermatogenesis (Sutherland *et al.* 2015). An important advantage afforded by sRNA loading by RBPs, such as binding with either AGO2 or nucleophosmin1 (NPM1), is that such a protein-bound state would provide protection from luminal RNases (Wang *et al.* 2010, Hoy & Buck 2012). Currently however, little is known about the presence and/or abundance of RBPs in the epididymal lumen, or their potential for association with maturing spermatozoa. Rather, owing to pioneering studies conducted by Sullivan and colleagues, an alternative mode of sRNA delivery involving their encapsulation in extracellular vesicles, specifically epididymosomes, has attracted the balance of attention in this field (Belleannée *et al.* 2013, Sullivan & Saez 2013). Thus, while RBP-mediated sRNA transport remains a possibility within the epididymis, and worthy of further investigation, for the purpose of this review, here we will only focus on epididymosome-mediated inter-cellular communication, and bulk delivery of sRNA cargo to spermatozoa transiting the epididymis.

## The contribution of epididymosomes to the altered small RNA profile of epididymal spermatozoa

Epididymosomes are broadly defined as membrane bound extracellular vesicles (EVs) that are produced within the principal cells of the epididymal epithelium (Fig. 2C). Noting that epididymosomes have a diameter in the range of between 50 and 150 nm (Frenette *et al.* 2010) and contain an abundance of proteomic cargo typical of small EV subtypes (Girouard *et al.* 2011, Nixon *et al.* 2018), these entities are generally classified as exosomes (Lötval *et al.* 2014). However, epididymosomes do display some degree of heterogeneity, both in terms of their encapsulation of defined exosomal markers, such as CD9 and other tetraspanin partners, and also in their capacity to interact with target cells (Frenette *et al.* 2010), thus making definitive classification difficult (Sullivan 2015). Moreover, epididymosomes are delivered to the epididymal lumen via an apocrine secretory pathway that involves the formation of large blebs at the apical margin of the parent cell. After narrowing of the processes attaching these apical blebs to their parent cells, they eventually detach and subsequently fragment releasing their contents into the extracellular compartment (Hermo & Jacks 2002).

In the epididymis, bleb disintegration leads to the release of encapsulated epididymosomes, along with other cellular-derived content, into the epididymal lumen. Following their release, epididymosomes are able to interact with neighbouring epithelial cells to affect a form of paracrine regulation, thereby indirectly influencing sperm maturation via maintenance of the complex epididymal luminal milieu (Belleannée *et al.* 2013). Alternatively, epididymosomes are also brought into close apposition with luminal spermatozoa to exert a more direct influence on sperm maturation via delivery of fertility modulating cargo to these recipient cells (Sullivan & Saez 2013, Martin-DeLeon 2015). Since their initial discovery in the hamster (Yanagimachi *et al.* 1985), epididymosomes have been identified in the reproductive tract of a number of other species including the bull (Frenette & Sullivan 2001), mouse (Rejraji *et al.* 2006), ram (Ecroyd *et al.* 2004), cat (Rowlison *et al.* 2018) and human (Thimon *et al.* 2008). Furthermore, the interaction between epididymosomes and sperm is now a widely accepted means by which to deliver a complex proteomic and lipidomic payload to maturing spermatozoa (Frenette *et al.* 2002, Rejraji *et al.* 2006).

Exosome-mediated cell-to-cell communication is well characterised in many biological systems. In comparison however, research into this vesicle-dependent mechanism of molecular transport in the male reproductive tract is relatively scarce. Nevertheless, the balance of evidence indicates that epididymosome–sperm interactions are likely to be selective, with epididymosome proteins

having been shown to be preferentially transferred to the post-acrosomal domain of the sperm head (Nixon *et al.* 2018) before putatively being distributed in a bidirectional manner to the anterior region of the head and mid-piece of the flagellum (Frenette *et al.* 2005). This selectivity raises the prospect that the composition of the sperm plasma membrane and/or the epididymosomes themselves may influence the efficacy of their union, with specialised membrane micro domains known as lipid rafts having been implicated in the coordination of initial epididymosome–sperm interactions (Girouard *et al.* 2009). Accordingly, these micro domains are highly enriched in cholesterol and sphingolipid (Harroun *et al.* 2008) and serve to compartmentalize GPI-anchored proteins such as P25b and SPAM1 (Griffiths *et al.* 2008), which themselves have been implicated in epididymosome–sperm adhesion. In this context, the proteolytic release of GPI-linked proteins from the sperm lipid rafts leads to a significant reduction in the efficacy of epididymosome cargo transfer to the sperm cells (Girouard *et al.* 2009). Moreover, it has been shown that extensive remodelling of the lipid membrane of both epididymosomes and spermatozoa occurs along the length of the epididymis, with the epididymosome lipid bilayer being characterised by progressive increases in rigidity, and the sperm plasma membrane displaying the opposing trend of increased fluidity (Rejraji *et al.* 2006). Such changes are believed to favour the transfer of lipids from epididymosomes to sperm, a finding supported by the uptake of labelled lipids (DilCl2) from bovine epididymosomes to spermatozoa *in vitro* (Caballero *et al.* 2013).

Despite this evidence, the absence of major epididymosome membrane-bound proteins in spermatozoa suggests that epididymosome–sperm interactions do not encompass a complete fusion event (Gatti *et al.* 2005). Similarly, it has been suggested that endocytic uptake, one of the principal routes for exosome internalisation in somatic cells (Mulcahy *et al.* 2014), is unlikely to occur in mature spermatozoa (Jones *et al.* 2013). This is because spermatozoa lack both the machinery needed to internalise exogenous molecules via endocytosis and the lysosomal organelles to which endocytosed cargo are typically targeted (Jones *et al.* 2013). Rather, epididymosome adherence may be followed by the creation of a transient fusion pore and subsequent release of the epididymosome once delivery of their cargo is complete. Accordingly, proteomic analyses of epididymosomes, and spermatozoa themselves, have identified a myriad of complementary trafficking proteins (including SNARE proteins, Ras-like proteins and dynamins) (Girouard *et al.* 2011, Nixon *et al.* 2018) that could regulate this form of intercellular communication. This model may, in part, account for why a portion of epididymosomes persist in seminal fluids rather than being completely absorbed by spermatozoa within the duct. While there is currently limited

functional evidence linking any of these trafficking proteins to a role in sperm–epididymosome interaction, immunoelectron microscopy analysis has confirmed the presence of stalk-like projections forming at the interface of epididymosome–spermatozoa contact (Nixon *et al.* 2018). Similar ultrastructural features are also witnessed between spermatozoa and oviductosomes (extracellular membrane vesicles released into the oviductal fluid) (Al-Dossary *et al.* 2015), raising the prospect of conserved mechanisms for facilitating cargo delivery between spermatozoa and the different populations of extracellular vesicles they encounter during their journey to the site of fertilisation. Consistent with these findings, the transfer of epididymosome proteins can be significantly inhibited by antibody masking of MFGE8, a protein that possesses a RGD recognition motif implicated in integrin/ligand interactions that proceed cellular fusion (Raymond *et al.* 2009, Nixon *et al.* 2018). Regardless of their mechanism of uptake, epididymosome interactions culminate in significant compositional modifications of the sperm membrane architecture and cytosolic domains (Griffiths *et al.* 2008, Schwarz *et al.* 2013). Further, the demonstrated ability of epididymosomes to encapsulate their ferried cargo within a protective lipid bilayer in the epididymal lumen identifies epididymosomes as an attractive vehicle to transport sRNA to transiting epididymal sperm.

Indeed, recent studies have begun to provide proof of principle that epididymosomes are capable of trafficking sRNA cargo to spermatozoa (Reilly *et al.* 2016), with deep sequencing analysis revealing a global increase in sRNA abundance in sperm following co-incubation with epididymosomes *in vitro* (Sharma *et al.* 2018). Hence, a role for epididymosomes in modulating the sRNA profile of sperm is possible (Eaton *et al.* 2015). Accordingly, analysis of epididymosomes sampled from specific segments along the length of the epididymis has revealed substantial modification to their sRNA cargo, many of which correspond with changes documented in spermatozoa, thereby providing correlative evidence to implicate these vesicles in influencing the epigenome of sperm during epididymal transit (Reilly *et al.* 2016, Hutcheon *et al.* 2017, Sharma *et al.* 2018) (Fig. 2B).

Analysis of the sRNA cargo of epididymosomes documented numerous sRNA species, including the miRNAs and tRFs as well as those sRNAs derived from snRNAs, snoRNAs and rRNAs (Hutcheon *et al.* 2017). Interestingly, the sRNAs encapsulated in epididymosomes are distinct to many of those harboured by their parent cells, a finding that indicates selective sRNA packaging by parent cells for subsequent release via epididymosomes (Belleannée *et al.* 2013, Reilly *et al.* 2016); a characteristic that is shared among exosomes released from non-reproductive tissues (Guduric-Fuchs *et al.* 2012). It is of particular interest to note here however, that mouse epididymosomes do not harbour substantial amounts of the piRNA sRNA

class (Hutcheon *et al.* 2017), and co-incubation of epididymosomes and spermatozoa *in vitro* does not result in substantial piRNA transfer (Sharma *et al.* 2018). Thus, it is unlikely that epididymosomes account for the elevated accumulation of this sRNA class documented in mature cauda spermatozoa (Fig. 2A). Rather, the identification of proteins involved in piRNA production in cauda spermatozoa invites speculation that sperm may harbour precursor piRNA transcripts that can be used as substrates by pathway machinery proteins for piRNA production (Hutcheon *et al.* 2017).

Taken together, these data build a case for a role of epididymosomes in establishing the sperm sRNA profile during epididymal transit. However, as evidenced by work on the piRNA class of sRNA, the extent of this contribution potentially differs between each sRNA species. A recent perspective piece further extrapolated this role by suggesting that epididymal derived extracellular vesicles and their sRNA cargo are important in the transgenerational inheritance of environmental effects (Eaton *et al.* 2015). This role was suggested to involve epididymosomes facilitating communication between the exposed epididymal epithelium and the spermatozoa migrating through the epididymal lumen. Indeed, successive research has revealed that exposure to a number of environmental stressors can influence the sperm sRNA profile (Marczylo *et al.* 2012, Fullston *et al.* 2013, Gapp *et al.* 2014) and that epididymosomes are potential mediators of this altered sRNA accumulation profile (Sharma *et al.* 2016, Rompala *et al.* 2018).

### The role of epididymosomes in mediating an altered small RNA profile in spermatozoa in response to the environment

It is now well established that the sRNA profile of sperm is altered in response to a changed environment. One of the first studies to examine this phenomenon utilised a high-throughput sequencing approach to reveal an increased accumulation of a subset of miRNAs, in addition to an opposing reduced accumulation of the piRNA class of sRNA, in mouse spermatozoa post-exposure to early life trauma in the form of unpredictable maternal separation in combination with unpredictable maternal stress (Gapp *et al.* 2014). Further, the offspring of exposed fathers displayed distinct behavioural phenotypes, including heightened fear and depressive-like behaviours. Via microinjection of the total RNA pool of sperm of traumatised mice into fertilised oocytes, the causal relationship of these responses was demonstrated. Such an approach successfully recapitulated the offspring phenotype; a demonstration that implicated sperm as the vector, and their RNA cargo as the causative agents, in this intergenerational inheritance model (Gapp *et al.* 2014). This demonstration also subsequently led to a series of

studies that further explored this paradigm using other stress models (Table 1). Collectively, from the studies summarised in Table 1, it is now apparent that an array of paternal stressors, ranging from dietary perturbation (e.g. high fat, low protein), chronic psychological stress, ethanol consumption, environmental pollution and cigarette smoke exposure, can each differentially alter the sRNA profile of sperm of exposed males (Li *et al.* 2012, Marczylo *et al.* 2012, Rodgers *et al.* 2013, Saavedra-Rodriguez & Feig 2013, Gapp *et al.* 2014, Metzler-Guillemain *et al.* 2015, Paris *et al.* 2015, de Castro Barbosa *et al.* 2016, Donkin *et al.* 2016, Fullston *et al.* 2016, Murashov *et al.* 2016, Rompala *et al.* 2016, Sharma *et al.* 2016, Short *et al.* 2016, 2017, Benito *et al.* 2018, Dickson *et al.* 2018, Ingerslev *et al.* 2018, Rompala *et al.* 2018). Further, some of these studies went on to demonstrate that an altered sperm sRNA profile contributed to altered phenotypic outcomes in the offspring post fertilisation. For example, independent work by de Castro Barbosa *et al.* (2016) and Fullston *et al.* (2016) has provided evidence that fathers exposed to a high-fat diet produce spermatozoa with altered sRNA profiles and that the resulting offspring of these males express overt metabolic disturbances. However, an altered sperm sRNA profile is not always associated with negative phenotypic outcomes, with exercise intervention in mice leading to an anxiolytic behavioural phenotype in offspring (Short *et al.* 2017).

Whilst the majority of these studies have focused on defining the role of sperm-borne sRNAs as '*information carriers*' in intergenerational inheritance models, determining the mechanism(s) by which the sperm sRNA profile is altered under conditions of stress remains an important area of investigation. Interestingly, altered abundance of several sperm miRNA and tRF sRNAs, including *miR-375*, *miR-191-5p*, *miR-467e*, tRF-Gly-GCC and tRF-Glu-CTC, has been directly attributed to epididymosome-mediated transferral mechanisms via *in vitro* co-culture experiments (bolded text in Table 1 and; Reilly *et al.* 2016, Sharma *et al.* 2018). However, more compelling causal links implicating epididymosomes in this phenomenon have been established in recent reports that utilised the mouse model to study chronic ethanol consumption. Specifically, the tRF cargo of epididymosomes from mice exposed to this insult demonstrated equivalent alterations to those observed in their spermatozoa (Rompala *et al.* 2018). By providing the first evidence of a concomitant modification of the sRNA cargo of both epididymosome and sperm in response to paternal insult (Rompala *et al.* 2018), this study builds on earlier reports alluding to the importance of epididymosomes in conveying an altered tRF profile to the spermatozoa of males placed on a low-protein diet (Sharma *et al.* 2016).

To begin to explore whether conditions of stress converge to elicit a common dysfunction in epididymosome trafficking, or alternatively, whether

**Table 1** Small RNA present in mammalian cauda sperm and reported as significantly differentially accumulated in response to insult.

<b>Insult</b>	<b>Animal model</b>	<b>Increased accumulation</b>	<b>Decreased accumulation</b>	<b>ES</b>	<b>F1</b>	<b>Offspring phenotype</b>	<b>References</b>		
High-Fat Diet	Species: Mouse Strain: C57BL/6	<i>miR-30a-5p<sup>t</sup></i> <i>miR-126a-3p<sup>t</sup></i> <i>miR-126a-5p</i> <i>miR-133b-3p</i> <i>miR-135b-5p</i> <i>miR-136-5p</i>	<i>miR-141-5p</i> <i>miR-143-3p<sup>t</sup></i> <i>miR-145a-5p</i> <i>miR-337-3p</i> <i>miR-367a-3p</i> <i>miR-376b</i>	<i>miR-669d</i> <i>miR-669n</i> <i>miR-669l</i> <i>miR-672</i> <i>miR-879</i> <i>miR-1967</i> <i>miR-1969</i>	n/a	x	Metabolic and reproductive phenotype	Fullston et al. (2016)	
High-Fat Diet	Species: Rat Strain: Sprague-Dawley	<i>let-7c-5p<sup>t</sup></i> <i>piR-025883</i> <i>piR-015935</i>	<i>tRNA1684-GluCTC.5</i> <i>tRNA11370-GluCTC.5</i>	<i>miR-92a</i> <i>miR-139-3p</i> <i>miR-150</i> <i>miR-184-3p</i> <i>miR-412</i> <i>miR-467</i> <i>miR-466a-3p</i> <i>miR-466b-3-3p</i> <i>miR-669c</i> <i>miR-293-5p</i> <i>miR-880-3p</i> <i>piR-036085</i>	n/a	✓	Metabolic phenotype	de Castro Barbosa et al. (2016)	
Obesity	Species: Human	<i>piR-31115</i> <i>piR-31445</i> <i>piR-33044</i> <i>piR-35407</i>	<i>piR-36246</i> <i>piR-45120</i> <i>piR-57942</i>	<i>piR-35548</i> <i>piR-36378</i> <i>piR-36707</i>	n/a	n/a	n/a	Donkin et al. (2016)	
Low protein diet	Species: Mouse Strain: FVB/NJ	<i>miR-103</i> <i>tRF-Gln-TCC</i> <i>tRF-Gly-CCC</i> <i>tRF-Cly-CTT</i> <i>tRF-Lys-CTT<sup>t</sup></i>	<i>tRF-His-GTC<sup>t</sup></i> <b>tRF-Gly-GCC</b>	<i>let-7c<sup>t</sup></i> <i>let-7f<sup>t</sup></i> <i>let-7e<sup>t</sup></i> <i>let-7b<sup>t</sup></i> <i>miR-16a</i> <i>tDR-SerACA</i>	<i>miR-98<sup>t</sup></i> <i>miR-883</i> <i>tRF-Phe-GAA</i> <i>tRF-Arg-TCT</i>	n/a	n/a	n/a	Sharma et al. (2016)
Chronic Ethanol	Species: Mouse Strain: C57BL/6	<i>miR-102<sup>t</sup></i> <i>miR-10b</i> <b>miR-299t<sup>t</sup></b>	<b>tDR-Glu-CTC</b> <i>tDR-His-GTC<sup>t</sup></i> <i>tDR-Tyr-GTA</i> <i>tDR-Pro-ACC</i> <i>tDR-Thr-AGT</i> <i>tDR-Pro-AGG</i> <i>tDR-Glu-TTC</i> <i>tDR-Gln-CTG</i> <i>tDR-Pro-TGG</i>	<i>miR-16a</i> <i>tDR-SerACA</i> <i>tDR-Leu-TAA</i> <i>tDR-Ile-AAAT</i> <i>tDR-Lys-TTT</i> <i>tDR-Ser-CCA</i> <i>tDR-Ser-TGA</i>	✓	n/a	Same exposure previously shown to impart stress hypersensitivity	Rompala et al. (2016)	
Chronic variable stress	Species: Mouse Strain: C57BL/6; 129 F1 hybrid	<i>miR-29<sup>t</sup></i> <i>miR-30a<sup>t</sup></i> <i>miR-30c<sup>t</sup></i> <i>miR-32</i> <i>miR-193</i>	<i>miR-29<sup>t</sup></i> <i>miR-30a<sup>t</sup></i> <i>miR-30c<sup>t</sup></i> <i>miR-32</i> <i>miR-193</i>	–	–	n/a	Behavioural phenotype – HPA stress axis dysregulation	Rompala et al. (2013)	
Trauma – unpredictable maternal separation	Species: Mouse Strain: C57BL/6			<i>miR-100-5p</i> <i>let-7a-5p<sup>t</sup></i> <i>let-7b-5p<sup>t</sup></i> <i>let-7c-5p<sup>t</sup></i> <i>let-7d-5p<sup>t</sup></i> <i>let-7e-5p<sup>t</sup></i> <i>let-7f-5p<sup>t</sup></i> <i>let-7g-5p</i> <i>let-7i-5p</i> <i>miR-9-5p</i> <i>miR-10a-5p<sup>t</sup></i> <i>miR-10b-5p<sup>t</sup></i> <i>miR-15b-5p</i> <i>miR-21a-5p</i> <i>miR-26a-5p</i> <i>miR-26b-5p<sup>t</sup></i>	<i>miR-100-5p</i> <i>miR-103-3p</i> <i>miR-125a-5p</i> <i>miR-125b-5p</i> <i>miR-128-3p</i> <i>miR-135a-5p</i> <i>miR-148a-3p</i> <i>miR-182-5p</i> <i>miR-183-5p<sup>t</sup></i> <b>mir-191-5p</b> <i>miR-194-5p</i> <i>miR-200b-3p</i> <i>miR-200c-3p</i> <i>miR-204-5p<sup>t</sup></i> <i>miR-342-3p</i>	n/a	x	Altered behavioural and metabolic responses in offspring	Gapp et al. (2014)

(Continued)

Table 1 Continued.

Insult	Animal model	Increased accumulation	Decreased accumulation	ES	F1	Offspring phenotype	References	
Stress-corticosterone administration	Species: Mouse Strain: C57BL/6	<i>miR-26b<sup>t</sup></i> <i>miR-38t<sup>t</sup></i> <i>miR-144t<sup>t</sup></i> <i>miR-190bt<sup>t</sup></i>	–	<i>miR-34c-5p<sup>t</sup></i> <i>miR-449at<sup>t</sup></i>	n/a	n/a	Hyper anxiety-like behavioural phenotype	
Early life trauma	Species: Mouse Strain: CD-1	–		n/a	✓	Induced anxiety and sociability defects detected from previous study (Saavedra-Rodriguez & Feig (2013))	Dickson et al. (2018)	
Smoking	Species: Human	<i>let-7a-2-3p</i> <i>miR-7</i> <i>miR-30Ct<sup>t</sup></i> <i>miR-146b-3p</i> <i>miR-34Q</i> <i>miR-305</i> <i>miR-509-5p</i> <i>miR-519d</i> <i>miR-550a</i> <i>miR-550b</i> <i>miR-576-3p</i> <i>miR-23b-3p</i> <i>miR-33b-3p</i> <i>miR-92a-2-3p</i> <i>miR-126-3p<sup>t</sup></i> <i>miR-129-3p<sup>t</sup></i> <i>miR-181d</i> <i>miR-186</i> <i>miR-221-3p</i> <i>miR-10bt<sup>t</sup></i> <i>miR-33b</i> <i>miR-106a</i> <i>miR-155</i> <i>miR-183t<sup>t</sup></i> <i>miR-30Ct<sup>t</sup></i> <i>miR-30e</i> <i>miR-34Ct<sup>t</sup></i> <i>miR-92a</i> <i>miR-98t<sup>t</sup></i> <i>miR-99bt<sup>t</sup></i> <i>miR-122</i> <i>miR-124</i> <i>miR-134a</i>	<i>miR-576-5p</i> <i>miR-933</i> <i>miR-944</i> <i>miR-1246</i> <i>miR-1267</i> <i>miR-1285</i> <i>miR-3145-3p</i> <i>miR-4513</i> <i>miR-4748</i> <i>miR-451</i> <i>miR-5217c</i> <i>miR-548g</i> <i>miR-548n</i> <i>miR-564</i> <i>miR-762t<sup>t</sup></i> <i>miR-1246</i> <i>miR-3467-3p</i> <i>miR-205t<sup>t</sup></i> <i>miR-208a</i> <i>miR-222</i> <i>miR-223</i> <i>miR-361t<sup>t</sup></i> <i>miR-29c<sup>t</sup></i> <i>miR-363t<sup>t</sup></i> <i>miR-425</i> <i>miR-433</i> <i>miR-449c</i> <i>miR-450a</i> <i>miR-465a</i> <i>miR-500</i> <i>miR-503</i>	n/a	n/a	Not investigated	Marczylo et al. (2012)	
Environmental pollution	Species: Human			<i>miR-340-3p</i> <i>miR-517c</i> <i>miR-3679-5p</i>	n/a	n/a	Not investigated	Metzler-Guillemain et al. (2015)
X-ray irradiation	Species: Mouse Strain: CD1				n/a	n/a	Not investigated	Paris et al. (2015)

Long term exercise	Species: Mouse Species: C57BL/6J	<i>miR-145</i> <i>miR-147</i> <i>miR-183<sup>t</sup></i> <i>miR-196a<sup>t</sup></i> <i>miR-346</i> <i>miR-21</i> <i>miR-431</i> <i>miR-483-3p</i> <i>miR-19b-2</i> <i>miR-190b<sup>t</sup></i>	<i>miR-762<sup>t</sup></i>	<i>miR-221</i>	n/a	n/a	More susceptible to effects of high fat diet	Murashov <i>et al.</i> (2016)
Exercise	Species: Mouse Strain: C57BL/6	<i>miR-145</i> <i>miR-133a-1</i> <i>miR-133a-2</i> <i>miR-455</i> <i>miR-223-3p</i> <i>miR-3653-3p</i> <i>piR-2750<sup>t</sup></i> <i>piR-28160</i> <i>tDR-Pseudo-ACG.C.2</i>	<i>miR-1834</i>	n/a	n/a	n/a	Anxiolytic behavioural phenotype	Short <i>et al.</i> (2017)
Endurance training	Species: Human Strain: C57BL/6	<i>miR-143-3p<sup>t</sup></i> <i>piR-11690</i> <i>piR-26632</i> <i>tDR-Lys-CTT.4<sup>t</sup></i> <i>miR-132-3p</i> <i>miR-132-5p</i> <i>miR-212-3p</i> <i>miR-212-5p</i>	-	<i>piR-17444</i>	n/a	n/a	Not investigated	Ingerslev <i>et al.</i> (2018)
Environment enrichment	Species: Mouse Strain: C57BL/6				n/a	n/a	Improved cognition	Benito <i>et al.</i> (2018)

Underlined sRNA species signifies altered accumulation of sRNA was validated by a second technique (commonly real time-quantitative PCR). Bold sRNA indicate species shown to be transferred from epididymosomes to sperm via *in vitro* co-incubation experiments. ES column: studies that found a parallel change in the sRNA cargo of isolated epididymosomes (ES) under conditions of stress (✓, yes; ✕, no; n/a, not investigated). F1 column: studies that examined the sperm sRNA profile of offspring sperm from exposed fathers (✗, found no parallel change in offspring sperm sRNA profile; ✓, found similar alteration in offspring sperm sRNA profile to that established in fathers; n/a, did not investigate). Note: threshold of significance varied between studies included in this analysis (*P*-value cut off range <0.1–0.05).

<sup>t</sup>Denotes sRNA species reported in more than one of the studies summarised.

each stress drives unique alterations to the composition of the total sperm sRNA population, we surveyed the curated lists of sRNA profiles documented across multiple stress models (Table 1). Perhaps expectedly, this analysis revealed that each stress model displayed a divergent combination of changes to the sRNA profile of stressed sperm. Notably however, this analysis also identified a specific subset of sRNA species that have been reported to differentially accumulate in response to more than one environmental insult. Illustrative of this, among the 17 studies summarised in Table 1, 30 sRNAs, including 28 miRNAs repeatedly returned an altered change in abundance post-exposure of sperm to multiple environmental insults (denoted by <sup>t</sup> in Table 1). As a cautionary note, we emphasise that each miRNA has the ability to modulate the abundance of many numerous, functionally unrelated protein-coding transcripts, and thus, it is important to not over-interpret the significance of similar changes in sRNA abundance (i.e. the same sRNA and a matching abundance trend) post exposure to multiple stressors. Further, we acknowledge the variations in documented sperm sRNA changes arising from the application of similar stress exposure models in independent laboratories. The extent to which such variability is attributed to differences in experimental design, methodologies and/or model species/strains employed remains to be determined. Notwithstanding these caveats, our interrogation of the available literature provides preliminary evidence to suggest that each imposed stress results in a unique signature of altered sRNA abundance in exposed sperm. Considering this information, it is tempting to speculate that rather than simply being a legacy of reproductive tract dysfunction, these changes may hold some adaptive value (Rechavi & Lev 2017). Indeed, this hypothesis has recently been promulgated on the basis of studies in invertebrate models, where it is suggested that males are able to transmit an adaptive advantage to subsequent generations (Rechavi *et al.* 2011, Wang *et al.* 2017). However, whether this paradigm is applicable to complex organisms, such as vertebrates, remains to be elucidated.

While epididymosomes potentially mediate the transfer of sRNA from the epididymal soma to maturing spermatozoa, these vesicles and their harboured cargo are produced within the epididymal epithelial cells. Therefore, under stress conditions, the physiology of the epididymal epithelial cells is likely altered; a physiological change that could initiate a series of biochemical events which result in an altered sRNA population being produced and packaged into the epididymosomes generated by stressed cells. Currently, this is not an active area of research focus within this field, however, with each individual insult demonstrated to elicit a unique change to the sRNA profile of sperm (Table 1), it is likely that the production and packaging of sRNAs into epididymosomes within the stressed

epididymal epithelial cell, differs with each individual insult. To date, this paradigm has been considered in the context of chronic stress, where it has been suggested that exposure to elevated corticosterone modulates the expression of the glucocorticoid receptor, and this in turn promotes aberrant epididymal epithelial cell signalling (Pang *et al.* 2017). The dysregulation of intracellular signalling is, in part reflected by an altered miRNA production profile and packaging into epididymosomes, thus putatively driving equivalent changes to the sperm sRNA profile (Pang *et al.* 2017). Whilst plausible, this proposed model obviously requires experimental validation.

## Concluding remarks

The sRNA profile of spermatozoa is now recognised as an important epigenetic contributor to early embryo development and subsequent offspring health. Indeed, the specific reshaping of this profile that occurs during epididymal transit is required to produce a mature spermatozoon that is not only capable of initiating fertilisation but also supporting early embryonic development and the generation of healthy offspring. However, the plasticity of the sRNA profile under conditions of stress and the consequences of an altered sRNA profile are becoming increasingly apparent. This therefore places heightened emphasis on the need to understand the mechanisms responsible for altering the sRNA landscape of sperm of stressed males. Among the most attractive mechanisms for modulating the global sRNA landscape of sperm under normal physiological conditions, and those encountered during episodes of stress, are epididymosomes. These specialised extracellular vesicles have been implicated in a novel form of intercellular communication, which is capable of dramatically altering the proteomic, lipidomic and epigenetic landscape of the maturing spermatozoon. What remain less certain are the underlying biological processes that drive alterations of the sRNA cargo of epididymosomes. Further research in this field promises to advance our currently limited understanding of the legacy of exposure to environmental insult on future generations and to inform rational therapeutic intervention strategies.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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## CHAPTER TWO: ORIGINAL RESEARCH ARTICLE

# The reproductive toxicant acrylamide modulates the mouse epididymal proteome to drive alterations to the sperm epigenome and dysregulate embryo development

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<b>Abstract</b>	Paternal exposure to environmental stressors elicits distinct changes to the sperm sncRNA profile; modifications that have significant post-fertilization consequences. Despite this knowledge, there remains limited mechanistic understanding of how paternal exposures modify the sperm sncRNA landscape. Here, we report the acute sensitivity of the sperm sncRNA profile to the reproductive toxicant, acrylamide. Further, we traced the differential accumulation of acrylamide-responsive sncRNAs to coincide with sperm transit of the proximal (caput) segment of the epididymis, wherein acrylamide exposure altered the expression of several transcription factors implicated in the expression of acrylamide-sensitive sncRNAs. We also identified extracellular vesicles secreted from the caput epithelium in relaying altered sncRNA profiles to maturing spermatozoa, the implications of which manifest in the form of dysregulated gene expression during early embryonic development. These data provide mechanistic links to account for how environmental insults can alter the sperm epigenome and compromise the transcriptomic profile of early embryos.
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## CHAPTER OVERVIEW:

Paternal exposure to a range of environmental and lifestyle factors elicits distinct changes to the sperm sncRNA profile which influence paternal mediated transmission of offspring phenotypes. This investigation identified sperm-derived sncRNAs as epigenetic contributors to the zygote and provided a causal link between an altered sperm sncRNA profile following exposure to environment stress and a compromised embryonic developmental trajectory. Such findings prompted us to explore the origin of these epigenetic changes, with the balance of evidence pointing to the epididymis as being the conduit for relaying environmentally acquired sncRNA signals to the male germline. This stage of post-testicular maturation is of fundamental importance in the functional transformation of the sperm cell and is accompanied by a dynamic remodelling of the sperm sncRNA profile. While evidence has implicated this stage of sperm maturation, studies focusing on exposure to paternal insult solely during epididymal sperm transit are limited. Hence, the study outlined in this chapter examined the impact of exposure to the xenobiotic, acrylamide during sperm epididymal transit. Therefore, in focusing on the mechanism(s) of sperm sncRNA profile change we limited our investigation to the epididymal soma and luminal constituents these cells produce. Data within this chapter confirms the responsiveness of the sperm sncRNA profile to environmental insult during epididymal transit. Further, we established the global proteomic response to acrylamide exposure of epididymal epithelial cells, revealing an increased expression of seven transcription factors predicted to regulate the transcription of the acrylamide sensitive miRNAs. In seeking to establish the conduit facilitating this dialogue to the male germ line we identified an increase in miRNA in epididymosomes, comparable to the modifications that occur in the mature sperm.

Additionally, we explored sperm-borne sncRNAs as the paternal factor contributing to the variable levels of reproductive toxicity associated with acrylamide exposure. Indeed, acrylamide exposure while sperm reside in the epididymis confers a stress signal to the cells that leads to an increase in post-implantation embryo loss. As mentioned above, exposure to acrylamide during this maturation phase is also accompanied by an altered miRNA profile. Interestingly, consequent embryo loss is not apparent when acrylamide administration is timed to expose spermatozoa while spermatocytes in the testis. Hence, we generated 2-cell embryos using these two populations of spermatozoa and surveyed the embryo transcriptome. This analysis revealed a global down regulation of miRNA gene targets in the embryos fertilised by spermatozoa exposed during epididymal transit compared to the spermatocyte exposed embryos, highlighting acrylamide responsive miRNAs in driving gene dysregulation in the embryo. Overall, evidence from within this chapter revealed the responsiveness of the sperm sncRNA profile to acrylamide and identified a novel mechanism to account for this modification.

**TITLE:** The reproductive toxicant acrylamide modulates the mouse epididymal proteome to drive alterations to the sperm epigenome and dysregulate embryo development

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**SHORT TITLE:** Acrylamide alters epididymal soma proteome and sncRNA profile of sperm

**KEYWORDS:** acrylamide, embryo development, epididymis, proteome, small non-protein-coding RNA, spermatozoa, sperm maturation

**ABSTRACT:**

Paternal exposure to environmental stressors elicits distinct changes to the sperm sncRNA profile; modifications that have significant post-fertilization consequences. Despite this knowledge, there remains limited mechanistic understanding of how paternal exposures modify the sperm sncRNA landscape. Here, we report the acute sensitivity of the sperm sncRNA profile to the reproductive toxicant, acrylamide. Further, we traced the differential accumulation of acrylamide-responsive sncRNAs to coincide with sperm transit of the proximal (caput) segment of the epididymis, wherein acrylamide exposure altered the expression of several transcription factors implicated in the expression of acrylamide-sensitive sncRNAs. We also identified extracellular vesicles secreted from the caput epithelium in relaying altered sncRNA profiles to maturing spermatozoa, the implications of which manifest in the form of dysregulated gene expression during early embryonic development. These data provide mechanistic links to account for how environmental insults can alter the sperm epigenome and compromise the transcriptomic profile of early embryos.

## **INTRODUCTION:**

In what has become a well-established paradigm, mature spermatozoa harbor a highly complex and dynamic repertoire of small non-protein-coding regulatory RNAs (sncRNAs) that are delivered to the oocyte upon fertilization. Despite the modest size of the total sperm RNA pool (equating to ~20-100 fg), in comparison to the endogenous pool of oocyte RNA (~0.5 ng), multiple observations have shown that the former actively contribute to early embryo development to influence offspring phenotypes<sup>1, 2, 3, 4, 5, 6</sup>. Moreover, unlike the genetic code, epigenetic information carriers, such as sncRNAs, are susceptible to dynamic compositional change in response to the environment<sup>7, 8, 9</sup>. Indeed, the sperm sncRNA profile displays considerable plasticity in response to paternal exposure to a variety of lifestyle and environmental stressors with such profile alterations increasingly linked to significant post-fertilization consequences<sup>7, 8, 10, 11</sup>. What remains less certain however, is the mechanistic basis by which pervasive environmental exposures alter the sperm sncRNA profile to elicit reproductive perturbations.

In seeking to resolve this question, spermatozoa are known to encounter at least two potential windows of vulnerability during their protracted journey to functional maturity within the male reproductive tract. The first coincides with the spermatogenic cycle within the seminiferous tubules of the testes. This developmental phase encompasses both meiotic divisions and an elaborate cytodifferentiation process (spermiogenesis), which together transform spermatogonial stem cells into highly morphologically specialized mature spermatozoa<sup>12</sup>. Notably, the significant remodeling and condensation of the chromatin architecture during spermiogenesis renders spermatozoa transcriptionally silent, and thus, incapable of initiating a direct translational response to environmental insult<sup>13, 14</sup>. Similarly, the prospect that environmental stressors influence the composition of the testicular sncRNA profile within developing sperm cells is incongruous, as alteration of the testicular sncRNA profile, which exerts stringent control over spermatogenesis, would be expected to result in overt morphological defects or attenuation of sperm production<sup>15, 16, 17</sup>. In direct contrast to the

testes, the epididymis has long been recognized as fundamentally important for the functional transformation of the migrating spermatozoa<sup>18, 19</sup>. More specifically, the dynamic remodeling of the sperm sncRNA profile has recently been added to the already extensive catalogue of biochemical alterations that sperm experience during epididymal transit<sup>20, 21, 22, 23</sup>; with at least a subset of epididymal acquired sncRNAs having been implicated in the support of normal embryonic development<sup>5</sup>. Accordingly, the balance of evidence now rests with the epididymis as being the conduit for relaying environmentally acquired signals to the male germline<sup>24, 25</sup>. Furthermore, extracellular vesicles (EVs) generated by the epididymal soma have been implicated in the delivery of molecular signals that significantly influence the final sncRNA profile of mature spermatozoa<sup>9, 26</sup>. As the sire of extracellular vesicle biogenesis, it follows that epididymal epithelial cells are likely responsible for orchestrating responses to environmental insult; a response involving compositional change to both the RNA and protein cargo selected for extracellular vesicle packaging and subsequent delivery to spermatozoa<sup>27, 28</sup>. Although attractive, experimental evidence to substantiate this model is currently lacking.

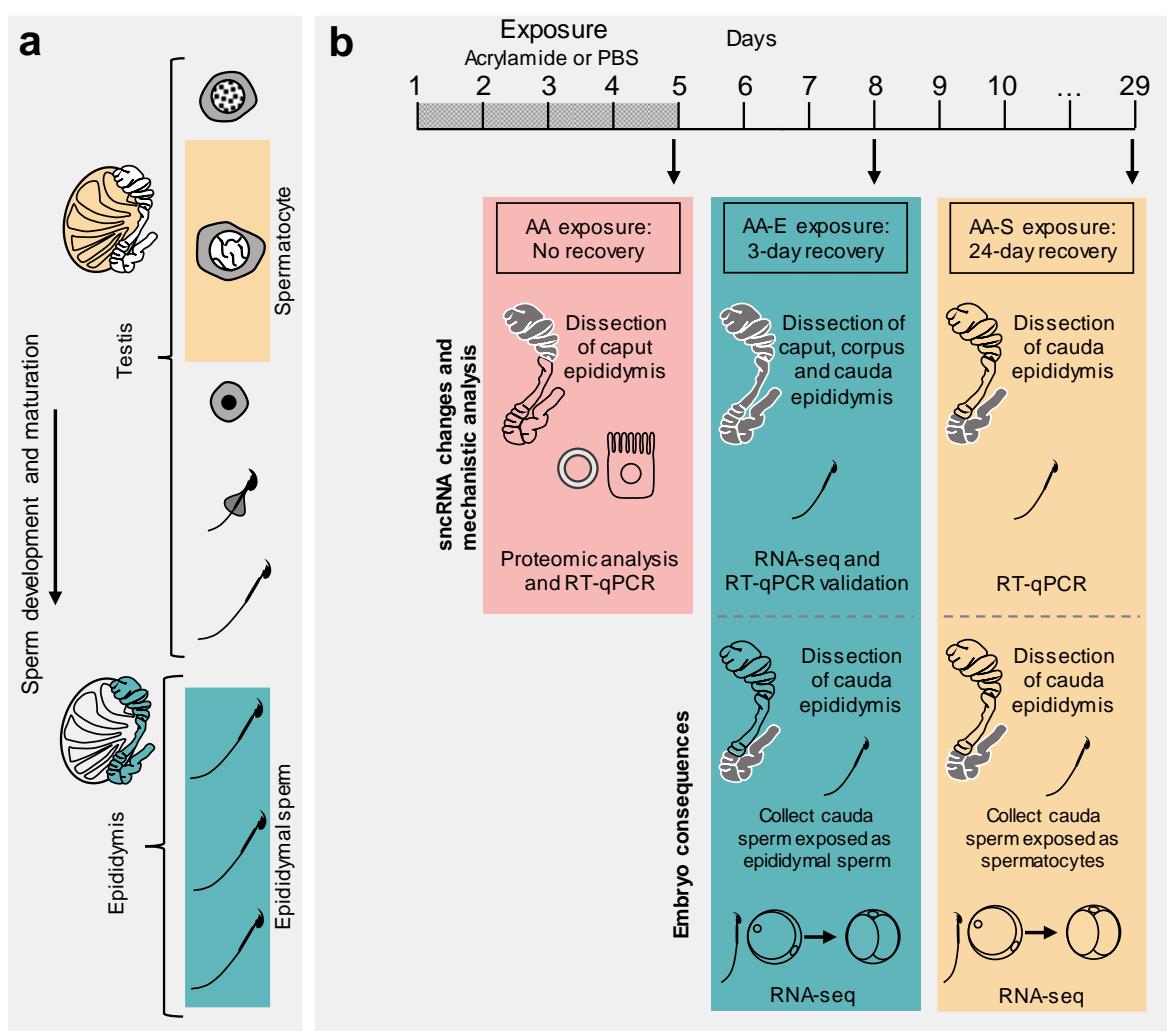
To address this knowledge gap, we utilized a tractable xenobiotic exposure regimen to assess the chain of cause and effect between altered epididymal epithelium cell signaling and modification to the sperm sncRNA profile. Specifically, mice were administered acrylamide using an established exposure regimen that elicits variable levels of reproductive toxicity dependent on the stage of development at which spermatozoa encounter the challenge<sup>29, 30, 31</sup>. Thus, despite harboring a comparable degree of DNA damage, a phenotype of embryonic developmental failure (i.e. embryo resorptions), was only observed in sperm that encountered the acrylamide challenge during epididymal, (AA-E) as opposed to testicular (AA-S) development<sup>29</sup>. This curious result raises the prospect that AA-E spermatozoa carry an additional ‘stress signal burden’, not present in AA-S sperm, and further, that this ‘molecular burden’ may be in the form of an altered sncRNA profile. Accordingly, acrylamide exposure was timed to coincide with sperm epididymal residence. Post stress regimen application, RNA-seq was therefore utilized to determine the extent of alteration to the sncRNA profile of

exposed spermatozoa, which are unable to support post-implantation embryo development. Quantitative mass spectrometry was subsequently employed to profile the global proteomic response of the epididymal soma, a response that potentially drives the alteration of the sperm sncRNA profile of exposed animals. This tractable model of xenobiotic exposure was additionally used to assess the contribution of sperm-delivered sncRNAs to the post fertilization outcomes characteristic of epididymal acrylamide exposure. To achieve this, we surveyed the high molecular weight transcriptomic profile of early embryos fertilized with either AA-E or AA-S spermatozoa, focusing on the gene targets of the acrylamide responsive sncRNAs.

## RESULTS:

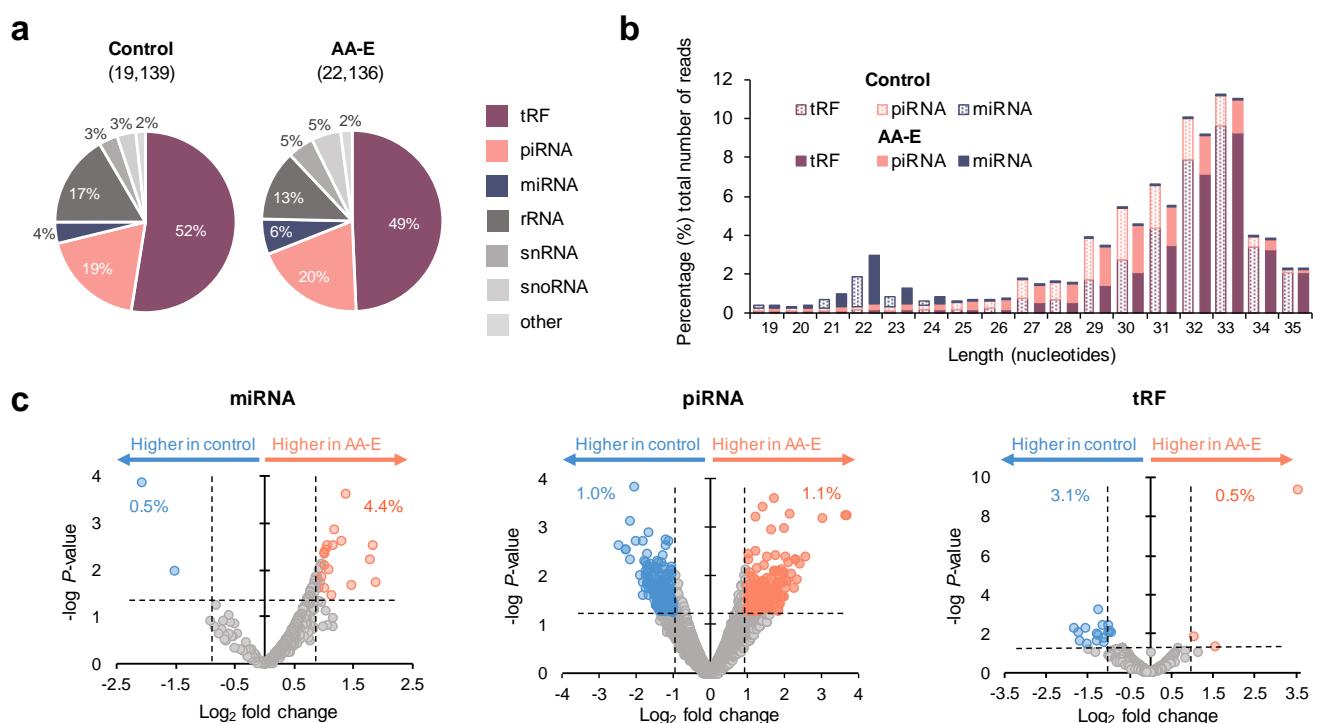
### **The small RNA profile of epididymal spermatozoa is altered by acute acrylamide exposure**

To determine the impact of acute acrylamide exposure on the sncRNA profile of mouse spermatozoa, adult males were exposed to acrylamide or vehicle alone (PBS: control) via intraperitoneal injection for five consecutive days (Fig 1). Three days after the final injection, mature spermatozoa were isolated from the cauda epididymis (AA-E) in preparation for sequencing of the sncRNA fraction. This strategy enabled the identification of 19,139 and 22,136 unique sncRNA sequencing reads for control and AA-E mice, respectively. Consistent with previous findings<sup>11, 21, 32</sup>, the sncRNA profile of control and AA-E spermatozoa was dominated (52% and 49%, respectively) by the transfer RNA-derived RNA fragment (tRF; 30-35-nt) species of sncRNA (Fig 2a; Supplementary Fig 1a). The sncRNA size distribution profile was comparable between both samples, with accumulation peaks corresponding to the microRNA (miRNA; 21-23-nt) and tRF species of sncRNA (Fig 2b), as reported previously<sup>32</sup>. However, despite these sncRNA size and species similarities, negative binomial exact assessments revealed that AA-E exposure altered the abundance of 2.5% (569) of all identified sncRNAs.



**Figure 1: Experimental Design.** **a** The development of the male germ cell, encompassing both the morphological maturation of the sperm cell within the testes and their subsequent functional maturation within the epididymis are depicted. The highlighted cell populations represent those stages of sperm development that were targeted by the acrylamide exposure regimens used in this study. **b** Mice were administered acrylamide (25 mg/kg bw/day) or vehicle alone (PBS; control) for five consecutive days via intraperitoneal (i.p.) injection. Mice were euthanized 2-3 h following the final injection (AA exposure) for isolation of epididymal epithelial cells and extracellular vesicles for mechanistic analysis. Alternatively, to obtain populations of spermatozoa exposed to acrylamide at different stages of development, mice were euthanized either three or twenty-four days following the final injection. Collection of spermatozoa three days following the final injection yielded a population of cauda spermatozoa exposed to acrylamide while exclusively residing in the epididymis (AA-E). This population of spermatozoa correspond to those subjected to RNA-sequencing (RNA-seq) and RT-qPCR validation experiments. By contrast, the collection of spermatozoa twenty-four days following the final acrylamide injection captured a population of cauda spermatozoa that had been exposed while developing in the testis as spermatocytes (AA-S). For two-cell embryo gene expression studies, cauda spermatozoa were isolated from animals in both the AA-E and AA-S exposure groups and prepared for *in vitro* fertilization.

Further, responsiveness to acrylamide exposure was particularly evident for the miRNA class, which increased in their profile representation from 4.0% in the control to 6.0% in the AA-E sperm samples (Fig 2a; Supplementary Fig 1b). This corresponded to 4.9% of the detected miRNAs exhibiting altered abundance in AA-E, compared to control spermatozoa (Fig 2c). In comparison, only 2.1% of piwi-interacting (piRNA) and 3.6% of tRF sncRNAs were determined to change in their abundance across these two samples (Fig 2c). Notably, of the 4.9% of miRNAs with altered abundance, the majority (15 out of 17 altered miRNAs) displayed increased abundance in response to acrylamide exposure.



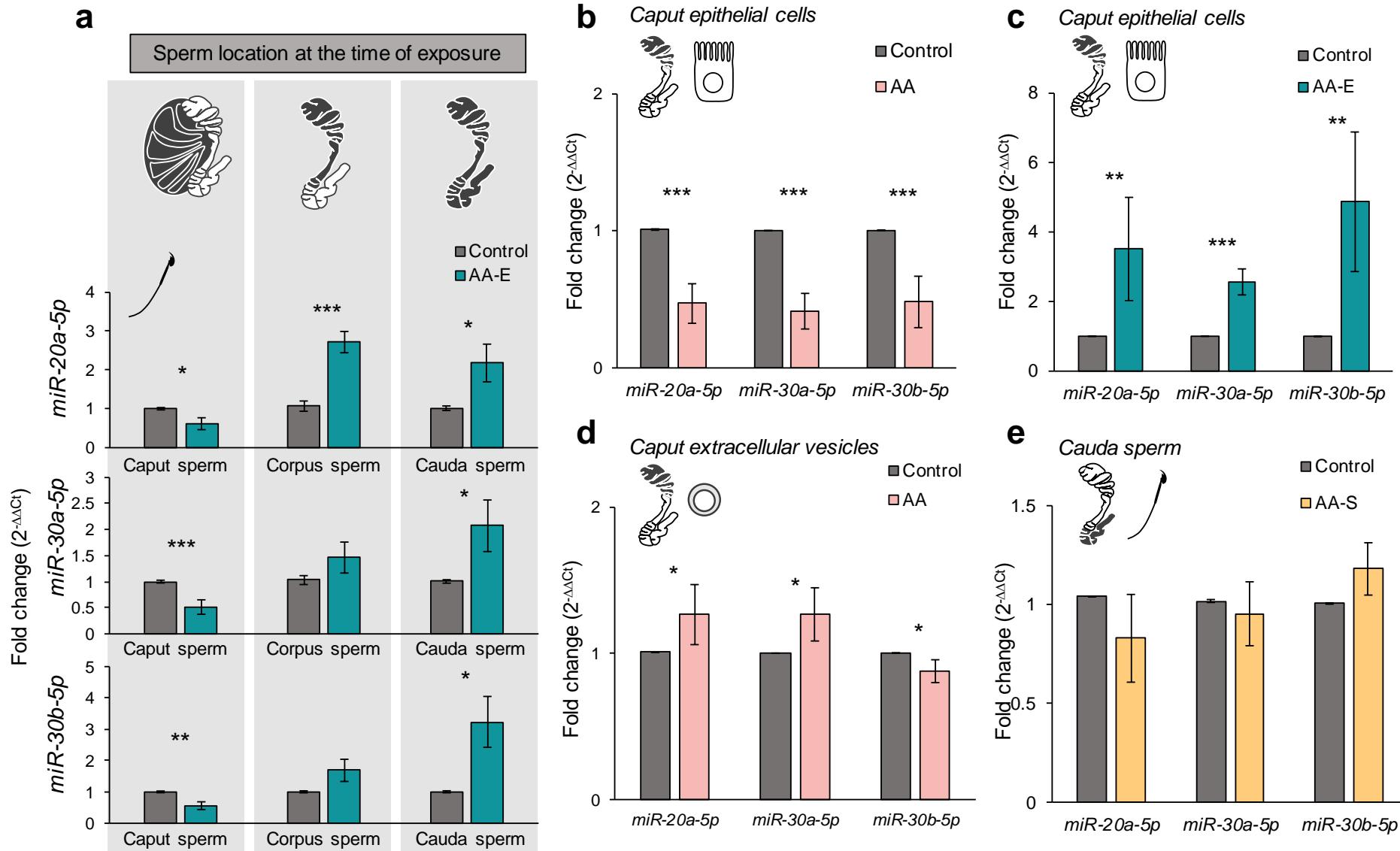
**Figure 2: Acute exposure to acrylamide during sperm epididymal transit (AA-E) alters the sperm small non-coding RNA (sncRNA) profile. a** Contribution of the major sncRNA classes to the global sncRNA landscape of mature control and AA-E cauda sperm populations. **b** Depiction of the proportion of microRNA (miRNA), Piwi-interacting RNA (piRNA) and transfer RNA derived fragment (tRF) sncRNAs contributing to the total number of reads for sequences of each length (19-35 nucleotides pictured). **c** Volcano plots highlight the log<sub>2</sub> fold change (x-axis) and -log P-value (y-axis) of identified miRNA, piRNA and tRF sncRNA transcripts in populations of mature spermatozoa following AA-E exposure. Dotted lines depict threshold values for significance of increased (orange) and decreased (blue) accumulation (i.e. -2 ≤ fold change ≥ 2 and P-value ≤0.05)

An independent cohort of animals was utilized to experimentally validate the sequencing identified abundance trends for eight selected sncRNAs. RT-qPCR readily confirmed the abundance trends for the eight selected sncRNAs across the control and AA-E cauda spermatozoa samples (Supplementary Fig 1c). Given that the miRNAs were the species of sncRNA most significantly affected by acrylamide exposure, and together with recent evidence supporting a role for miRNAs gained during epididymal transit in the regulation of early embryonic gene expression<sup>5,33</sup>, our focus was directed towards the miRNAs for further investigation.

#### **Acrylamide induced alterations to the sperm microRNA profile originate in the caput epididymis**

To identify the epididymal segment where alteration to the composition of the miRNA profile of sperm originated in response to acrylamide exposure, RT-qPCR was applied to quantify the abundance of three acrylamide responsive miRNAs, namely *miR-20a-5p*, *miR-30a-5p* and *miR-30b-5p*, across the epididymis. RT-qPCR revealed each candidate miRNA was of lower abundance in AA-E sperm than in control sperm isolated from the caput epididymis (Fig 3a, LHS). Whilst this is effectively the reciprocal trend to that in cauda spermatozoa (Fig 3a, RHS), the majority of sperm sampled from within the caput region were exposed to acrylamide during the final stages of testicular development (Fig 3a, top)<sup>34, 35</sup>. By contrast, in those sperm recovered immediately downstream, in the corpus segment (and therefore subjected to acrylamide during transit of the caput segment), a similar trend of increasing miRNA accumulation occurred; albeit only reaching a statistically significant change in the case of *miR-20a-5p* (Fig 3a). Based on these collective data, we infer that the soma of the caput epididymis is likely responsible for conveying an altered profile of sncRNAs to the transcriptionally inert sperm population harbored within the luminal environment of this segment of the tract

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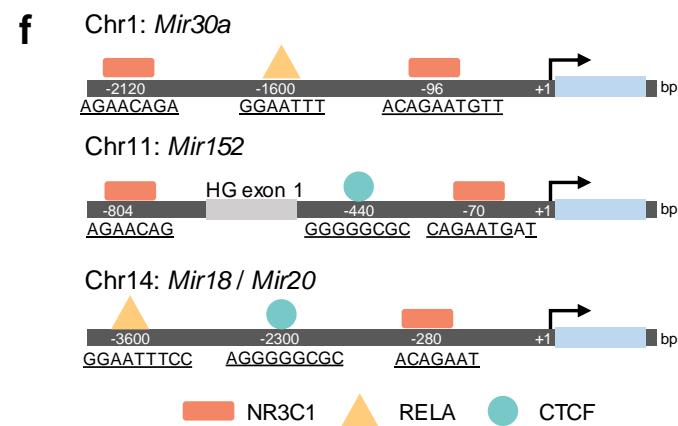
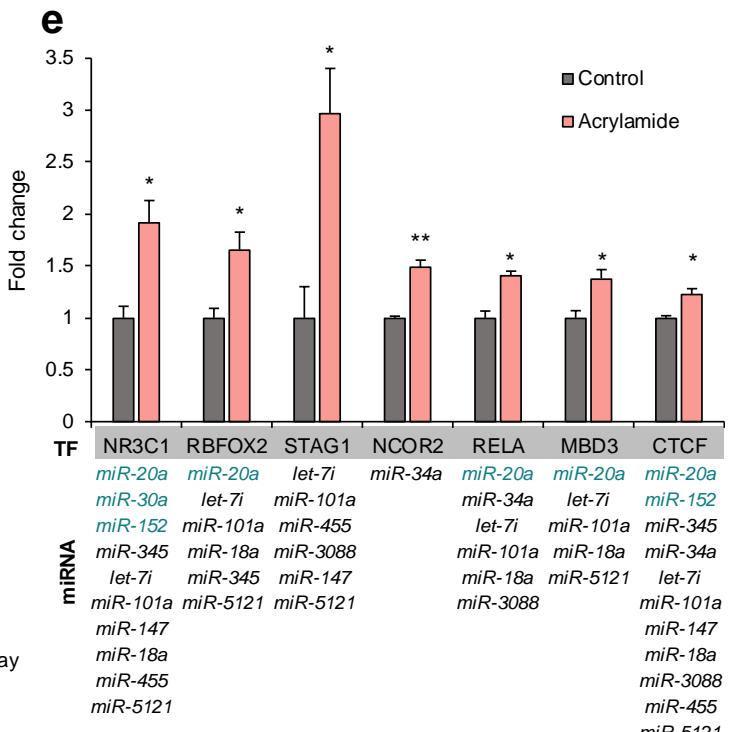
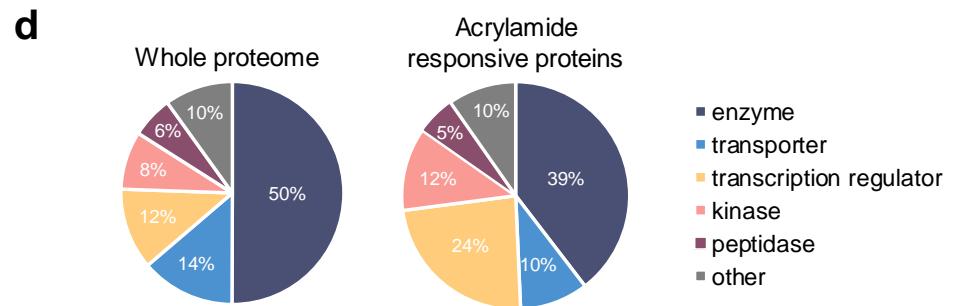
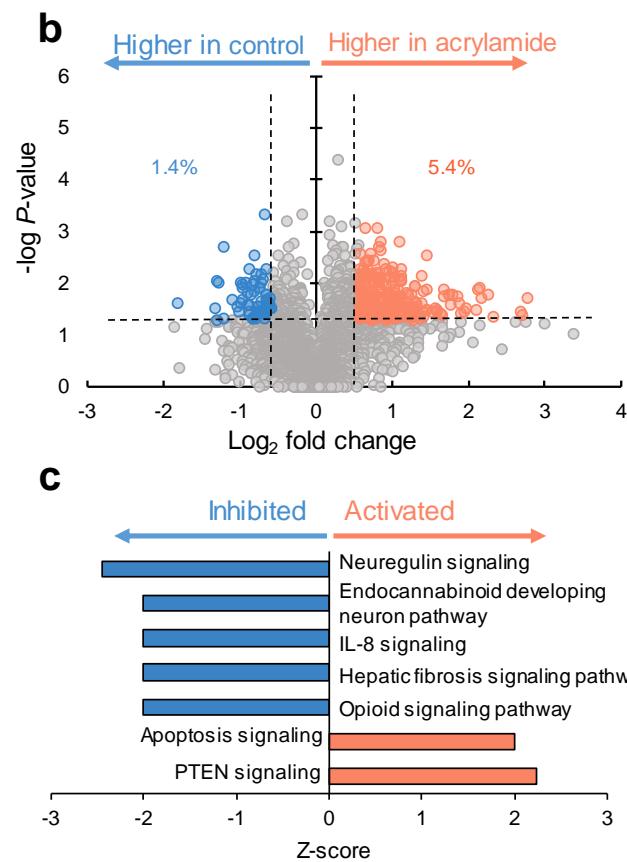
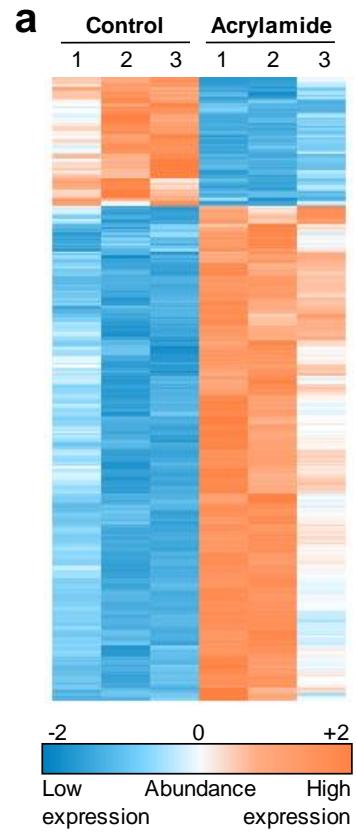
**Figure 3: Acrylamide-induced sperm microRNA (miRNA) changes originate in the caput epididymis.** Three miRNAs were selected from our validated candidates for examination of abundance in the epididymis of control and acrylamide exposed males. **a** RT-qPCR of miRNA fold change in spermatozoa sampled from the caput, corpus and cauda epididymal segments following acrylamide exposure. Timed collection meant that populations of cauda spermatozoa were exclusively exposed during their passage through the epididymis (AA-E). Consequently, populations of spermatozoa isolated from the more proximal epididymis were exposed to acrylamide at different locations along their developmental journey (as depicted by the region of dark grey shading within the male reproductive tract in the schematic above the graphs). **b** RT-qPCR of miRNAs in epithelial cells isolated from the caput epididymis of mice on the final day of injection (AA exposure), or **c** three days following the final injection (AA-E exposure). **d** The abundance of miRNAs in extracellular vesicles isolated from the caput epididymis of mice exposed to control or acrylamide and sacrificed on the final day of injection. **e** Examination of miRNA candidates in mature cauda epididymal spermatozoa exposed to acrylamide while developing in the testis (AA-S). Experiments were performed with at least three biological replicates ( $n = 3$  mice per replicate). The U6 small nuclear RNA or *let-7b* (panel **d** only) were used as endogenous controls for normalization of miRNA expression. Data are presented as mean  $\pm$  SEM of fold change between control and acrylamide determined by  $\Delta\Delta Ct$  method. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ . Therefore, we next examined the abundance of the same three miRNAs within both the soma and EVs of the caput epididymis from mice culled 2-3 h after the final acrylamide injection (termed AA samples in Fig 3b, d). The elimination of a recovery period in this instance ensures an actively exposed epididymis to more accurately replicate the physiological status of the soma that sperm would have encountered during their transit of the proximal epididymal segment, prior to being collected as mature cells from the distal cauda epididymis (Fig 1). A highly significant ( $P \leq 0.001$ ) decrease in the abundance of the three assessed miRNAs was evident in caput epithelial cells isolated from AA mice (Fig 3b), while an elevated abundance of each candidate miRNA was documented in epithelial cells recovered from the caput epididymis of AA-E mice, compared to the controls (Fig 3c). This suggests a dynamic, and potential over-compensatory response of the epididymal epithelium post cessation of acrylamide exposure. In profiling the abundance of miRNAs within EVs isolated from the caput epididymis (Fig 3d), a significant increase in the abundance of two of the three assessed miRNAs (*miR-30a-5p* and *miR-20a-5p*) was revealed in EVs of AA mice compared to the EVs of control mice. This finding suggested that EVs could function as the vehicle to traffic miRNAs to the spermatozoa post acrylamide exposure, albeit the third miRNA (*miR-30b-5p*) displayed a reciprocal response (Fig 3d). Notably, no significant change in the abundance of the three assessed miRNAs was revealed in spermatozoa that were exposed to acrylamide within the testis (AA-S) (Fig 3e).

### Proteome profiling identifies a novel mechanism for the alteration of the epididymal microRNA landscape

To attempt to identify the likely mechanism(s) by which acrylamide exposure alters the miRNA landscape of epididymal spermatozoa, we examined the legacy of acrylamide exposure on caput epithelial cells reflected in the proteome (Fig 1). This measurement of protein quantity enabled direct assessment of the biochemical response of the epithelial cells to acrylamide insult. Specifically, tandem mass tag (TMT) spectrometry based quantitative analysis identified 4,405 proteins across both the control and AA epithelial cell populations (Supplementary Table 1). Based on a threshold of  $\pm 1.5$ -fold change ( $P \leq 0.05$ ) in abundance, we identified a total of 302 caput epithelial cell proteins (equating to 6.8% of all identified

proteins) displaying altered abundance across the control and AA samples (Fig 4a). Among these proteins, 240 were identified with increased expression, with the remaining 62 proteins displayed reduced expression (Fig 4b). These changes in protein expression were validated using targeted proteomic methods (parallel reaction monitoring; PRM, Supplementary Fig 2). Ingenuity Pathway Analysis (IPA) of the proteins with altered abundance identified seven enriched pathways as acrylamide responsive; consisting of five pathways predicted to be inhibited, and two pathways predicted to be activated by acrylamide exposure (Fig 4c). Notably, proteins assigned to the categories of ‘transcription regulator’ and ‘kinase’ were enriched in the inventory of acrylamide responsive proteins. Conversely, those proteins classified as ‘enzyme’ and ‘transporter’ were under-represented (Fig 4d).

Acrylamide exposure could potentially influence sperm miRNA content through modulation of a number of molecular pathways, including the; (i) transcriptional regulation of *MIR* gene expression (due to DNA methylation, histone modifications or transcription factor influences); (ii) efficiency of miRNA precursor transcript processing (i.e. the rate of miRNA production); (iii) selectivity of miRNA packaging into EVs, and; (iv) biogenesis and release of EVs and their uptake by spermatozoa. Given that the specific mechanisms underlying these processes remain largely unexplored in the male reproductive tract, we aligned our proteomic dataset to examine changes in the key pathways utilizing resources established from other cell lineages (see references within Supplementary Table 2). Broadly, this analysis did not reveal any major dysregulation of specific proteins associated with the biogenesis of miRNAs, or of EVs. Likewise, proteins known to be associated with orchestrating the packaging of miRNAs into EVs were not influenced by acrylamide exposure (Supplementary Table 2), which when taken together, strongly infer that these three mechanisms are not the molecular mechanism likely responsible for altering the miRNA profile of epididymal spermatozoa post acrylamide exposure. By contrast, proteins involved in the transcriptional regulation of *MIR* gene expression were readily identified in our proteomic data stemming from our assessment of the epididymal epithelial cell proteome following acrylamide exposure.



**Figure 4: Proteomic analysis identifies a novel mechanism of sperm sncRNA alteration**

**a** Heat map depicting the differentially expressed proteins detected in three biological replicates of control and acrylamide exposed caput epididymal epithelial cells. Z-transformed abundance values were clustered by Euclidean distance method with average linkage to generate hierarchical heat map clusters of proteins with differential abundance. Orange and blue shading indicates increased and decreased protein abundance, respectively. **b** Volcano plot depicting the  $\log_2$  fold change (x-axis) and  $-\log P$ -value (y-axis) of the 4,405 epididymal epithelial proteins identified. Thresholds of  $\pm 1.5$  fold change and  $P$ -value of  $\leq 0.05$  in TMT reporter ion intensity were implemented to establish the proteins differentially expressed in caput epithelial cells from acrylamide exposed males to control males **c** Canonical pathways related to protein abundance changes in acrylamide exposed caput epididymal epithelial cells. A Z-score of  $\pm 2$  is considered predictive of activation / inhibition of the identified pathway. **d** Classification of the different protein types of the whole proteome and the restricted subset of acrylamide responsive proteins **e** Relative abundance of seven transcription factors (TF) in caput epididymal epithelial cells following acrylamide exposure as identified by TMT reporter ion intensity. Data are represented as fold change relative to control. \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ . The table below depicts mature miRNAs (increased in abundance in AA-E sperm) that each corresponding TF is predicted to regulate at a gene level. Green text indicates those miRNAs experimentally validated by RT-qPCR. **f** Schematic of putative TF binding sites upstream of pre-miRNA sequences (bent arrow) or host gene (HG) transcription start site. Chromosomal sequence extending from 3.7 kbp upstream of respective pre-miRNA sequences (bent arrow; +1) is represented by the dark grey box. Colored shapes indicate approximate binding sites and binding motifs of respective TFs. Light grey boxes represent host gene exons for intragenic miRNAs.

The gene loci that encode the non-protein-coding transcripts from which miRNAs are processed are subject to similar transcriptional control to that of protein coding genes<sup>36, 37</sup>. Therefore, as for a protein-coding locus the transcriptional activity of *MiR* genes can be regulated via epigenetic modifications and/or the altered abundance of transcription factors<sup>38</sup>. This knowledge led to our subsequent exploration of protein machinery involved in directing epigenetic modifications at the transcriptional level, such as those proteins required to direct cytosine methylation or the modification of the histones, including proteins such as DNA methyltransferases and histone de/acetylases. Again, this did not reveal changes in the abundance of such protein machinery between control and AA treated caput epithelial cells (Supplementary Table 2). Interestingly, examination of transcription factor (TF) expression, did however reveal increased expression of specific members of numerous TF families in AA exposed epithelial cells, compared to the control (Fig 4e). Upon further survey of the curated literature via the TF-miRNA regulation resource (TransmiR v2.0)<sup>39</sup>, the expression of a number of TFs, including NR3C1, RBFOX2, STAG1, RELA, MBD3 and CTCF, which target multiple miRNAs of interest, were confirmed as being significantly elevated ( $P \leq 0.05$ ) in AA exposed,

as compared to control, caput epithelial cells (Fig 4e). These findings took on added significance in view of our demonstration that binding motifs were harbored by the genomic sequences immediately upstream of the candidate acrylamide responsive miRNA genes (Fig 4f; Supplementary Table 3); a genomic position which suggested their location within the putative promoter regions of the *MIR* gene of each miRNA of interest.

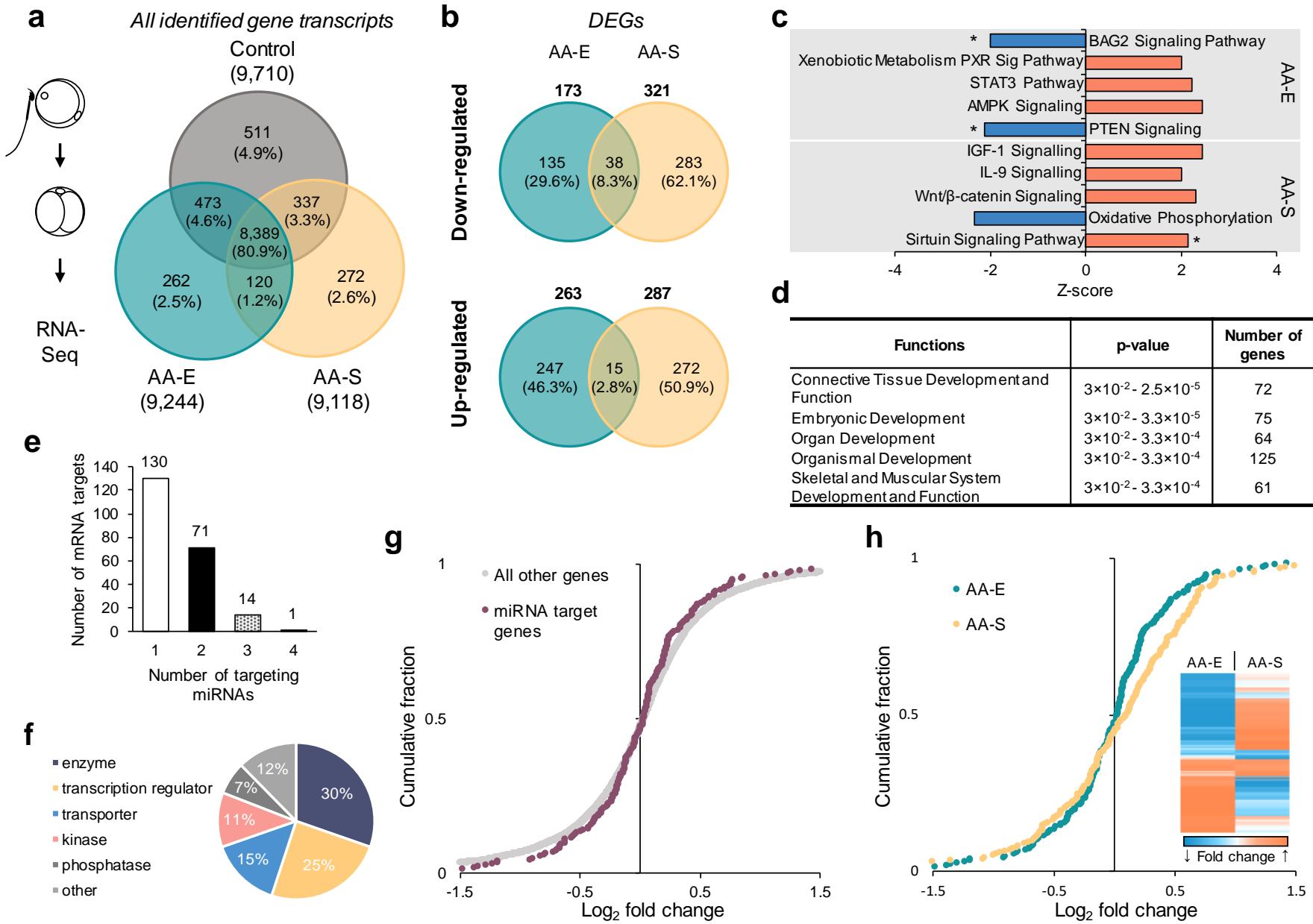
### **The influence of spermatozoa delivered small RNA exposed to acrylamide on preimplantation embryo gene expression**

Sperm-borne miRNA are delivered to the oocyte at the time of fertilization and are crucial regulators of early embryonic development<sup>4</sup>. Hence, we examined the impact of AA-E sperm miRNA changes on the early embryo using transcriptomic analysis. Two-cell embryos, fertilized by three different populations of spermatozoa, control and sperm from AA-E and AA-S exposed mice (Fig 1) were generated by *in vitro* fertilization (IVF). As mentioned previously, the population of AA-S spermatozoa harbor comparable levels of DNA damage to AA-E sperm, however, this DNA damage burden does not result in embryo resorptions<sup>29</sup>. Therefore, this approach allowed us to focus on the transcriptomic changes in embryos fertilized by spermatozoa exposed to acrylamide at different stages of their development, and to specifically identify gene expression dysregulation attributed to the altered miRNA profile of AA-E spermatozoa.

Scoring of sperm motility, fertilization rate, and embryo developmental progression, revealed no overt changes when compared to the control groups, aside from a subtle decrease in sperm motility (~6% decline) following capacitation in populations of AA-E sperm (Supplementary Fig 3a). However, this minor change did not translate to further functional defects, with sperm from exposed mice displaying comparable levels of capacitation, fertilization and the support of 2-cell embryo development compared to that of control spermatozoa (Supplementary Fig 3a-d).

Transcriptomic data from these 2-cell embryos revealed over 9,000 gene transcripts in each analyzed group, with the majority (80.9%) shared across the three assessed groups (Fig 5a). Approximately 6.3% of all identified gene transcripts were determined to be responsive to paternal acrylamide exposure (Fig 5a), with differentially expressed genes (DEGs) ( $P$ -value  $\leq 0.05$  and fold change  $\pm 2$ ), compromised of 173 and 321 down-regulated and 263 and 287 up-regulated genes, via comparison of the AA-E and AA-S datasets to that generated for control spermatozoa, respectively (Fig 5b). Interestingly, only a small portion of DEGs (8.3% and 2.8% for down- and up- regulated genes, respectively) showed a similar trend in altered expression in both the AA-E and AA-S embryos (Fig 5b; Supplementary Fig 3e, 2f). Substantiating this difference in gene expression between AA-E and AA-S embryos, compared to control embryos, the top five significant pathways enriched in the DEGs revealed no overlap (Fig 5c).

In focusing on gene expression differences between AA-E and control embryos, IPA analysis revealed a number of dysregulated networks mapping to physiological system and early embryonic development (Fig 5d). Next, we examined the extent of gene expression change driven by the delivery of an altered sperm miRNA profile in respect to embryos fertilized by AA-E sperm. We therefore utilized IPA to generate a list of experimentally confirmed gene targets of the 15 miRNAs increased in AA-E sperm. Indeed, 216 mRNA transcripts targeted by these miRNAs were also present in our embryo transcriptome dataset, of which 40% were identified as targets of more than one of the 15 miRNAs included in this analysis (Fig 5e). Additionally, the majority (55%) of these target mRNAs were classified as encoding either an ‘enzyme’ or a ‘transcription regulator’ (Fig 5f). In exploring the expression of these 216 target mRNAs in AA-E embryos, compared to control embryos, we found that miRNA target gene expression was modestly decreased in 2-cell embryos fertilized by AA-E sperm (Fig 5g). Further, in examining the log fold change of the 216 miRNA target genes in AA-E and AA-S embryos, compared to control embryos, an overall decrease in the transcript abundance of these miRNA target genes in AA-E embryos, compared to AA-S embryos, was evident (Fig 5h).



**Figure 5: The influence of paternal acrylamide exposure on preimplantation embryo gene expression.** **a** Venn diagram depicting total gene transcripts identified among three biological replicates for each group of embryos. **b** Venn diagrams demonstrating the overlap of differentially expressed genes (DEGs) between embryos generated with spermatozoa from each acrylamide exposure compared to the control. Fertilizing sperm were exposed either during epididymal transit (AA-E) or during the spermatocyte stage of sperm development in the testis (AA-S). **c** Top five enriched pathways identified in the list of DEGs between AA-E and control and AA-S and control embryos with significant Z-score. Bars with asterisks denote those with  $P$ -value  $\leq 0.05$  as determined by ingenuity pathway analysis (IPA). **d** Top functions in physiological system development and function networks identified in DEG list between control and AA-E embryos. **e** Experimentally observed mRNA targets of the increased miRNA in AA-E sperm (216 mRNA targets) distributed by the number of targeting miRNAs. **f** Classified protein types of the 216 mRNA targets as identified by IPA. **g** Cumulative distribution function (CDF) plot showing the  $\log_2$ -transformed expression differences for miRNA target genes (purple) and all other genes (grey) between AA-E and control embryos. **h** CDF plot showing expression differences for miRNA target genes in AA-E and AA-S embryos compared to control. Heat map (inset) depicting fold changes of the 216 miRNA target genes between AA-E and control and AA-S and control embryos.

This suggested the acrylamide responsive miRNAs, of increased abundance in AA-E sperm, are exerting regulatory control over their target gene transcripts, and further, that this regulatory influence does not occur in control or AA-S embryos. Moreover, the majority of the miRNA target transcripts displaying a negative fold change in AA-E embryos, exhibited either no change, or a modest increase in abundance in AA-S embryos, compared to control embryos (Fig 5h, inset). If the analysis was limited to the three validated acrylamide responsive miRNAs (Fig 3e), a similar decreased trend in target gene expression was revealed (Supplementary Fig 3g). Ultimately, we have identified a cohort of miRNA target genes with altered expression in early embryos that appear driven by the miRNA changes experimentally validated in AA-E sperm

## DISCUSSION:

Over the past decade, our understanding of male germ cell function has been transformed by the realization that mature spermatozoa not only harbor a substantial payload of sncRNAs, but that these regulatory molecules are relayed to the oocyte at the time of fertilization, and thereafter, influence the trajectory of embryo development<sup>1, 4, 40</sup>. Moreover, the sperm sncRNA landscape is dynamic, being appreciably remodeled as the cells undergo physiological maturation and in response to paternal experiences<sup>10</sup>. Indeed, it is now apparent that

environmental stressors as diverse as isolated traumatic events through to chronic nutritional perturbations can seemingly converge to alter the sncRNA cargo carried by the male germline and ultimately compromise the health of the offspring sired by exposed males<sup>7, 32</sup>. Whilst numerous studies have focused on establishing the causality of sncRNAs in the inheritance of altered phenotypic traits, comparatively, less is known regarding the mechanisms by which environmental stressor signals are communicated within somatic cells of the male reproductive system before being relayed to the germline in the form of an altered epigenetic cargo. In seeking to address this important knowledge gap, here we have exploited a tractable model of acute acrylamide challenge to reveal that the somatic epithelial cell lining of the caput epididymis is sensitive to environmental exposures. In responding to this challenge, the proteomic landscape of the epididymal soma is subtly recast, leading to an up-regulation of a subset of transcription factors responsible for regulating *MIR* gene expression, thereby altering the level of production of specific miRNAs. Accompanying this response, we identified equivalent changes in the miRNA packaging into EVs, and in the population of spermatozoa that encounter these EVs whilst residing in the lumen of the proximal caput epididymis at the time of acrylamide exposure. In linking this chain of cause and effect, embryos generated with the sperm of acrylamide exposed sires displayed altered gene expression profiles during early development, which could conceivably account for their susceptibility to developmental failure<sup>29, 31, 41</sup>.

As the model toxicant for this study, acrylamide is an organic compound produced industrially as a precursor to water-soluble thickeners and flocculation agents<sup>42</sup>. A prevalent additional source of human exposure arises from dietary consumption owing to the formation of acrylamide as a consequence of condensation (Maillard) reactions between amino acids and reducing sugars during the cooking of carbohydrate rich foods at temperatures exceeding 120°C<sup>43, 44</sup>. In addition to being classified as a neurotoxin and probable carcinogen, acrylamide has been irrefutably linked to reproductive toxicity<sup>45, 46</sup>. As might be expected, however, such deleterious reproductive pathologies vary in accordance with both the timing and the dose of

acrylamide exposure, with chronic exposure of male mice at human equivalent doses leading to DNA damage in the germline but no attendant loss of fertility<sup>47, 48</sup>. Such responses are accentuated by acute high dose acrylamide exposure; a treatment regimen that results in dominant lethality among sired embryos<sup>29, 31</sup>. Whilst we have previously linked this phenotype to elevated levels of sperm DNA damage, this etiology fails to entirely account for subsequent embryonic losses. Thus, spermatozoa exposed to acrylamide during testicular development (i.e. AA-S) produce numbers of viable embryos indistinguishable from those of vehicle-exposed controls. By contrast, embryonic losses (i.e. resorptions) averaging 73.5% are witnessed following fertilization with spermatozoa residing in the epididymal lumen (i.e. AA-E) during exposure. Notably, such differences manifest despite both populations of spermatozoa harboring equivalent levels of DNA damage and rates of *in vitro* fertilization<sup>29</sup>. These findings allude to the carriage of an alternative stress signal, with our findings firmly implicating altered profiles of sperm-borne sncRNA as a primary candidate.

In agreement with this model, the sncRNA profile of sperm subjected to acrylamide exposure during epididymal transit (AA-E) was clearly differentiated from that of their counterparts exposed during testicular development (AA-S). In particular, the AA-S exposure regimen failed to modulate the abundance of *miR-20a-5p*, *miR-30a-5p* or *miR-30b-5p* in the sperm, each of which were elevated in AA-E spermatozoa. Notably, in the somatic epithelium surrounding these cells within the caput epididymis these miRNAs displayed a decrease in abundance. However, the response of the caput epithelial cells to acrylamide challenge appeared dynamic, such that three days after the final acrylamide injection, the expression of acrylamide-responsive miRNAs responded in a reciprocal manner and displayed a significant increase, suggesting an over compensatory response upon the withdrawal of the acrylamide challenge. This finding encourages a future detailed appraisal of the legacy of paternal environmental exposure, with the potential to identify any persisting impacts following stress responses.

In an attempt to account for the selective impact of acrylamide on epididymal physiology, previous work has established that this toxicant is capable of permeating the blood-epididymis barrier to accumulate in the epithelial cells of the tract within as little as nine hours following administration<sup>49</sup>. Furthermore, cytochrome P450 2E1 (CYP2E1), the sole enzyme responsible for acrylamide metabolism, is abundantly expressed within the epithelium of the proximal epididymis<sup>29, 50</sup>. Compelling evidence from knockout mouse models and pharmacological inhibition studies has revealed that the reproductive toxicity of acrylamide is linked to the CYP2E1-mediated metabolism of this toxicant into the epoxide, glycidamide<sup>30, 51</sup>. Here, we demonstrate that either the biochemical detoxification of acrylamide, or the glycidamide by-product itself, are capable of eliciting changes in the proteome of the epididymal soma. This response draws interesting parallels with other cell types, such as those residing in the hippocampus and central nervous system, in which acrylamide also elicits demonstrable changes in the global proteome<sup>52, 53</sup>. Whilst we remain uncertain how this response is mediated, our data implicate TFs as a likely master regulator. Indeed, among the proteomic changes documented in acrylamide challenged caput epididymal epithelial cells, was a significant upregulation in the abundance of a number of TFs; including at least seven TFs that have been predicted to regulate the expression of the *MIR* genes from which the acrylamide responsive miRNAs altered in AA-E spermatozoa are processed.

Of the identified TFs, the glucocorticoid receptor TF, NR3C1, is of particular interest owing to its demonstrated role in orchestrating a molecular response to alternative forms of stress in numerous tissues<sup>54</sup>, including the male reproductive tract<sup>55</sup>. Consistent with the results presented here, elevated *NR3C1* gene expression has been reported following direct treatment of an epididymal epithelium cell line with corticosterone<sup>55</sup>. In extending these observations, the enhanced abundance of the NR3C1 TF that ensues post corticosterone challenge has been linked with an alteration to the protein and sncRNA cargo encapsulated within the EVs produced by epididymal cell lines<sup>26</sup>, as well as downstream changes in the abundance of a subset of sperm miRNAs, and ultimately, an altered offspring phenotype<sup>26, 56</sup>.

Undoubtedly, additional studies are needed to strengthen the causal link between altered epithelial cell NR3C1 abundance and the sperm-borne miRNA profile, however, such findings point to a convergent response to different paternal insults that may underpin changes to the epigenetic landscape of the maturing sperm cell. These collective data also identify the EVs produced by the epididymal epithelium as part of the intercellular communication network responsible for conferring paternal stress-associated signals between epididymal soma and recipient sperm<sup>9</sup>, and indirectly shaping the phenotype of the next generation<sup>26, 57</sup>. Likewise, our data implicates EVs in directing an acrylamide responsive sperm miRNA alteration. However, consistent with other observations<sup>9</sup> this form of communication does not account for the trafficking of all responsive sncRNA. Indeed, association with RNA-binding proteins, as seen with other mammalian cells<sup>58, 59</sup> and/or ‘nanotubes’ may represent additional trafficking mechanisms that contribute to shaping the sperm sncRNA profile following stress exposure<sup>60</sup>. It will, therefore, clearly be of value to refine our mechanistic understanding of how other sncRNA species (namely the tRF and piRNA sncRNA species) are modulated in response to paternal stress and trafficked to spermatozoa. While our proteomic analysis did not reveal significant changes to any known regulatory elements of the pathways that direct either the biosynthesis and/or processing of other sncRNA species, this does not discount the possibility that acrylamide leads to subtle changes in their functional activity, as opposed to abundance, of these molecules.

In any case, the altered profile of sncRNAs harbored by spermatozoa from AA-E, as opposed to the AA-S treatment regimen, provides a rational explanation for the curious differences in the developmental trajectory of embryos fertilized with these different sperm populations. Indeed, despite harboring an equivalent sub-lethal burden of DNA damage, both sperm populations retain the ability to navigate the female reproductive tract and fertilize the oocyte in both an *in vivo* and *in vitro* setting<sup>29</sup>. Thereafter, the oocyte appears to mount an effective repair response of paternal transmitted DNA damage<sup>61</sup>, such that embryos fertilized with AA-S sperm retain their full developmental potential. By contrast, the oocyte fails to

rescue the fate of AA-E fertilized oocytes, the majority of which ultimately succumb to post-implantation lethality. Contrary to other studies, which have alluded to the possibility of epigenetic lesions associated with the formation of glycidamide-DNA and/or glycidamide protamine adducts as primary causative agents underpinning dominant lethal mutations<sup>62</sup>, our experimental exposure regimen provides evidence that changes in the sperm sncRNA profile, uniquely acquired during their epididymal transit (AA-E), play a key role in contributing to this phenotype. This conclusion is strengthened by the demonstration of marked differences in the curated transcriptome of 2-cell embryos fertilized by spermatozoa of AA-E males versus the sperm of AA-S males. Indeed, embryos generated with AA-E spermatozoa were characterized by dysregulation of numerous genes mapping to key developmental pathways, potentially presaging the downstream arrest of embryonic development. By contrast, those spermatozoa bearing the legacy of an earlier AA-S challenge failed to elicit equivalent transcriptomic changes.

As anticipated, many of the dysregulated AA-E embryonic genes represent validated targets of the acrylamide responsive miRNAs relayed by AA-E spermatozoa and accordingly, follow reciprocal trends in abundance (i.e. the miRNAs displayed an increased abundance in AA-E spermatozoa, and their target gene transcripts downregulated in the embryo, compared to the control). Such findings accord with repeated demonstrations that sperm borne-miRNAs influence early embryonic gene expression by virtue of their ability to regulate maternal mRNA transcripts, with important implications for the trajectory of embryonic development and offspring phenotype<sup>5, 32, 63</sup>. This chain of cause and effect could also conceivably account for independent evidence of the down-regulation of a subset of genes in early embryos (i.e. 1-2 cell stage of development) generated from the spermatozoa of male mice subjected to an acute *in vivo* challenge with glycidamide<sup>62</sup>. Notably, in both studies, embryonic transcriptomic profiling was timed to precede the robust transcription of the mouse embryonic genome that occurs during the late 2-cell stage, findings which again emphasize that the deleterious impact

on embryonic gene expression is unlikely to be solely attributed to acrylamide / glycidamide induced sperm DNA damage<sup>64</sup>.

In summary, we report the first evidence that acute acrylamide exposure influences the sncRNA profile of mouse spermatozoa and provide important mechanistic links regarding both the origins and the consequences of an altered sperm sncRNA landscape. Whilst a key element of this response appears to be the unique sensitivity of the epididymal tissue to acute paternal insult, we also demonstrated the dynamic nature of this tissue in being able to rapidly respond following cessation of the stress. Such dynamic responses are echoed in studies of human spermatozoa, which have revealed the sncRNA profile they harbor can be altered within as little as a week of paternal exposure to dietary excursions (for example, a healthy versus high sugar diet)<sup>24</sup>. Whilst this is by no means a universal response<sup>26</sup>, it nevertheless highlights the importance of pre-conception male health, and may also identify a potential new avenue for the future development of targeted interventional therapies to combat the rising tide of male infertility<sup>65</sup>.

## **METHODS:**

### **Reagents**

All reagents used were of research or mass spectrometry grade, and unless specified, were obtained from Merck (Darmstadt, Germany) or Thermo Fisher Scientific (Waltham, MA, USA).

### **Ethics statement**

All experimental procedures were conducted with the approval of the University of Newcastle's Animal Care and Ethics Committee (ACEC; approval number A-2017-726), in accordance with national and international guidelines. Swiss mice were obtained from the University of Newcastle's Central Animal House and were housed under a controlled lighting regime (12 h light, 12 h dark) at 21-22°C and supplied with food and water *ad libitum*. Animals were acclimated for at least 1 week prior to treatment. Swiss mice (adult males of at least 8 weeks of age) were utilized for all experiments and were euthanized via CO<sub>2</sub> inhalation, prior to having their vasculature perfused with pre-warmed Tris-buffered saline (TBS) to eliminate blood contamination. The epididymides and vas deferens were dissected and separated from fat and connective tissue. The epididymides were carefully divided into three anatomical segments, corresponding to the caput, corpus and cauda epididymis and the segment of interest was prepared for isolation of spermatozoa, epithelial cells or extracellular vesicles as outlined below. All incubations and centrifugation were performed at 37°C, unless otherwise stated.

### **Acrylamide exposure regimen**

Male mice were administered acrylamide (25 mg/kg bw/day) or vehicle alone (control, phosphate buffered saline; PBS) via intraperitoneal injection once per day for five consecutive days. Mice were then euthanized at one of three different time points following the final injection (Fig 1). In preparation for isolation of epididymal epithelial cells and extracellular vesicles for mechanistic analysis, mice were culled 2-3h following the final injection (AA). The remaining two animal cohorts were euthanized to obtain populations of mature cauda

spermatozoa exposed to acrylamide at different developmental stages, coinciding with either epididymal transit (i.e., the AA-E cohort in which animals were euthanized three days following the final injection) or late stage testicular development as spermatocytes (i.e., the AA-S cohort in which animals were euthanized twenty-four days following the final injection).

### **Epididymal sperm isolation**

Mature sperm retrieval from the lumen of the caudal epididymis was facilitated by retrograde perfusion as previously described<sup>23</sup>. In contrast, caput and corpus spermatozoa were recovered by placing the dissected tissue into modified Biggers Whitten, and Whittingham (BWW) media<sup>66</sup> composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM CaCl<sub>2</sub>H<sub>2</sub>O, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>7H<sub>2</sub>O, 25 mM NaHCO<sub>3</sub>, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/mL penicillin, 5µg/mL streptomycin, 20 mM HEPES buffer, and 3.0 mg/mL bovine serum albumin [BSA]) (pH 7.4; osmolarity 300 mOsm/kg) and making multiple incisions in the tissue. Spermatozoa were allowed to ‘swim out’ for 15 min, before the sperm suspension was filtered through a 70 µm filter and centrifuged on top of a 28% Percoll density gradient (400 × g for 15 min). Recovered spermatozoa were washed in BWW media to remove any remaining Percoll and then subjected to RNA extraction.

### **RNA extraction and next-generation sequencing of the small RNA fraction of cauda spermatozoa**

Total RNA was extracted from populations of cauda spermatozoa isolated from control and acrylamide exposed male mice using a Direct-zol RNA MiniPrep Kit (Zymo Research Cooperation, Irvine, CA, USA) according to manufacturer’s instructions. This preparation of total RNA was pooled from six to ten animals to generate a single biological replicate (returning approximately 2.0 µg total RNA). One microgram from two such replicates was subjected to NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB, Ipswich, MA, USA) following the manufacturer’s recommendations at the Australian Genome Research Facility (AGRF;

Brisbane, QLD, Australia). The prepared libraries were sequenced at AGRF using an Illumina HiSeq-2500 RNA-seq platform as 50-bp single-end chemistry.

### **Bioinformatic analysis of small RNA sequencing datasets**

Raw sequences obtained from the AGRF were initially processed with CutAdapt (<https://cutadapt.readthedocs.io/>)<sup>67</sup> and FastQC Babraham Bioinformatics (/projects/fastqc/). Raw sequenced reads were trimmed of the adaptor and primer sequences. The remaining reads were scanned and trimmed to remove low-quality ends (defined as a base with a Phred quality score < 20). Trimmed reads shorter than 17 bases and low-quality sequenced reads (analyzed using FastQC) were discarded. Sequenced reads were then mapped against the *Mus musculus* reference genome 9 (NCBI37/mm9) and the RNACentral sequence database (January 2018, RNACentral) (<https://rnacentral.org/>) using alignment algorithms Bowtie<sup>68</sup> and Bowtie 2<sup>69</sup>. To align reads to the reference database, initially the default options of –n and –best were selected and reads producing single unique alignments were considered successfully mapped. Reads with more than one alignment or not successfully mapped were re-processed using Bowtie 2 with default settings and reads with a single valid alignment were considered successfully mapped and combined with the previously mapped reads. Any remaining unaligned reads were excluded from further analyses. Mapped reads were further processed using two internally developed Perl algorithms. Using the individual accession numbers the first algorithm queried the RNACentral database to sort each read by sncRNA species, while the second algorithm compiled and calculated the number of each unique sncRNA molecule across all samples in each treatment and respective controls. To assess the differential accumulation of sncRNA in sperm from acrylamide and control mice, data for both groups were imported into R Statistical Software<sup>70</sup> and analyzed using the edgeR package<sup>71, 72</sup>. A minimum count cut-off value of 10 reads was applied to determine the presence of individual RNAs in at least one sample group. Differential accumulation was calculated for each individual sncRNA using a negative binomial exact test between controls

and treated samples. The Benjamini and Hochberg's approach for controlling false discovery rate (FDR)<sup>73</sup> was applied to all results to adjust for multiple testing.

### **Purification of caput epididymal epithelial cells**

Spermatozoa were removed from the caput epididymis by placing the tissue in a droplet of BWW and making multiple incisions with a razor blade. Using methodology adapted from Kierszenbaum et al.<sup>74</sup> and as described previously<sup>75</sup>, epididymal epithelial cells were then isolated. Enrichment of epithelial cell populations was assessed by immunocytochemistry using a nuclear stain, 4',6-diamidino-2-phenylindole (DAPI) to identify contamination of spermatozoa. Upon confirmation of target cell enrichment, populations of isolated epididymal cells were used for proteomic profiling.

### **Epididymal epithelial cell protein digestion and labeling for comparative and quantitative proteomic analysis**

Epididymal epithelial cell preparations from control and acrylamide treated animals were pooled from five to six animals to generate a single biological replicate, with three such replicates being generated for analysis. Samples were prepared as previously described<sup>76, 77, 78, 79</sup>. In brief, thawed cell suspensions containing lysis buffer (100 µL of ice-cold 0.1 M Na<sub>2</sub>CO<sub>3</sub>; pH 11.3) supplemented with protease and phosphatase inhibitors (Complete EDTA free; Roche Holding SG, Basel, Switzerland) were probe tip sonicated at 4°C for 3 × 10 sec cycles (100% output power) prior to incubation at 4°C for 1 h. A bicinchoninic acid assay (Thermo Fisher Scientific) was conducted to determine the protein concentration of each sample. Protein solutions were diluted in urea (6 M urea, 2 M thiourea), reduced using 10 mM dithiothreitol (DTT) (30 min, room temperature) and alkylated using 20 mM iodoacetamide (30 min, room temperature, in the dark). Proteins were digested with Lys-C/Trypsin using 1:30 ratio of protease to total protein concentration, for 3 h at room temperature. The urea concentration was then reduced to below 1 M by addition of 50 mM tetraethylammonium bromide (TEAB; pH 7.8) and incubated at 37°C overnight. Lipids were precipitated using

formic acid (2% v/v final concentration), and peptide populations were purified using desalting columns (Oasis PRIME HLB; Waters, Rydalmer, NSW, Australia). Quantification of peptides was performed using fluorescent quantification (Qubit) and 100 µg of each sample was labeled using tandem mass tags (TMT) and comparative analyses was performed. (TMT 10plex labels; control 1 = 126, control 2 = 127N, control 3 = 127C, acrylamide 1 = 129N, acrylamide 2 = 129C, acrylamide 3 = 130N) (TMT-10plex; Thermo Fisher Scientific).

### **Tandem mass spectrometry (nanoLC-MS/MS) comparative and quantitative analyses**

Reverse phase nLC-MS/MS was performed on 11 HILIC enriched fractions using Q-Exactive HF-X Hybrid Quadrupole-Orbitrap MS coupled to a Dionex Ultimate 3000RS LC nanoflow high-performance liquid chromatography system (Thermo Fisher Scientific). Separation was then achieved using an in-house packed column, SGE MyCapLC Kit (Kinesis) 300 µm x 150 mm, employing a stepped linear gradient of acetonitrile (300 nL/min; 3-25%, 55 min; 25-60%, 70 min; 60-98%, 15 min). A Q-Exactive HF-X-MS System was operated in full MS/data dependent acquisition MS/MS mode (data-dependent acquisition). The Orbitrap mass analyzer was used at a resolution of 60,000 (FWHM) to acquire full MS with an m/z range of 300-1,650 and via incorporating a target automatic gain control value of  $3 \times 10^6$  and maximum fill times of 50 ms. The 15 most intense multiply charged precursors were selected for higher-energy collision dissociation fragmentation with a normalized collisional energy of 32. MS/MS fragments were measured at an Orbitrap resolution of 45,000 (FWHM) using an automatic gain control target of  $1 \times 10^6$  and maximum fill times of 120 ms.

### **Proteomic data processing**

Database searching of all raw files was performed using Proteome Discoverer 2.4 (Thermo Fisher Scientific). SEQUEST HT was used to search against the Uniprot *Mus musculus* database (25,260 sequences, downloaded 12<sup>th</sup> November 2019). Database searching parameters included up to two missed cleavages, a precursor mass tolerance set to 10 ppm and fragment mass tolerance of 0.02 Da and trypsin was designated as the digestion enzyme.

Interrogation of the corresponding reversed database was also performed to evaluate the false discovery rate of peptide identification using Percolator on the basis of q-values, which were estimated from the target-decoy search approach. To filter out target peptide spectrum matches over the decoy-peptide spectrum matches, a fixed false discovery rate of 1% was set at the peptide level. Protein lists were exported from Proteome Discoverer 2.4 as Excel files. The list of proteins was further refined to only include those with a quantitative value in all three replicates, and a minimum of two unique peptides, resulting in the identification of 4,405 proteins.

#### **Validation of quantitative protein accumulation in epididymal epithelial cells**

Validation of quantitative differences in protein abundance in epididymal epithelial cells detected by nLC-MS/MS was performed using the targeted mass spectrometry strategy, parallel reaction monitoring (PRM)<sup>80</sup>. Proteins selected for analysis included those highly abundant in our sample or displaying a significant difference in abundance following acrylamide exposure. Peptides were purified as described above and subjected to reversed phase chromatography using Dionex Ultimate 3000RSLC EasySpray 25 cm columns were used in combination with nano-ESI source. PRM was performed using Exploris 480 mass spectrometer (Thermo Scientific, Bremen, Germany). Methods optimized for collision energy, charge state, and retention times for peptides corresponding to proteins of interest, identified in the discovery TMT dataset. A full MS scan was performed with a resolution of 60,000 and targeted MS2 spectra were acquired using a PRM approach at a resolution of 15,000 employing a normalized AGC target of 100% and a maximum injection time of 120 ms. Raw data was analyzed using Skyline (MacCoss Lab, University of Washington)<sup>81</sup>, where the top six fragment ion intensities for individual peptide sequences were normalized to each sample's respective total ion count. Quantification was performed by comparing the normalized values between biological triplicates of control and treated cells using a Student's *t*-test.

### **Caput extracellular vesicle isolation**

Enriched populations of mouse caput extracellular vesicles were isolated as previously described<sup>82</sup>. Briefly, epididymal tissue was placed in a droplet of BWW media and luminal contents were released by making multiple incisions with a razor blade and allowing dispersion over 30 min. The luminal fluid suspension was then filtered through a 70 µm membrane and sequentially centrifuged with increasing velocity (500 × g, 2,000 × g, 4,000 × g, 8,000 × g, 17,000 × g) to eliminate cellular debris. The resultant supernatant was then layered onto a discontinuous OptiPrep gradient (40%, 20%, 10%, 5%) and ultracentrifuged at 100,000 × g for 18 h at 4°C. Following ultracentrifugation, twelve equivalent fractions were collected. Fractions 9 and 10 (the fractions at which extracellular vesicles readily partition away from other contaminants<sup>76, 82</sup>) were diluted in PBS and subjected to a final ultracentrifugation step (100,000 × g, 3 h, 4°C) before being subjected to RNA extraction.

### **Oocyte collection, *in vitro* fertilization and two-cell embryo culture**

Female 4 to 6-week-old Swiss mice were superovulated by intraperitoneal injection of 7.5 IU equine chorionic gonadotropin (eCG) and 7.5 IU human chorionic gonadotropin (hCG) 48 hours later (Provet, Sydney, NSW, Australia). Cumulus-oocyte complexes were retrieved from the distal oviductal ampullae thirteen hours after hCG injection and recovered in human tubal fluid (HTF) media, before being allocated into a droplet of HTF supplemented with 1.0 mM reduced glutathione (GSH) ready for *in vitro* fertilization (IVF). Oocytes retrieved from individual female mice were split into two groups for IVF by sperm from control or acrylamide exposed males. Spermatozoa were collected from the cauda epididymis by retrograde perfusion via the vas deferens as described above and simultaneously capacitated in modified BWW containing 1.0 mg/mL methyl-β-cyclodextrin for 45 min at 37°C under an atmosphere of 5% O<sub>2</sub>, 6% CO<sub>2</sub> in N<sub>2</sub>. A subset of this population of spermatozoa were assessed for phosphorylation of tyrosine residues to confirm capacitation-like changes in these cells, as previously described<sup>83</sup>. Two × 10<sup>5</sup> capacitated sperm were added to the oocyte containing HTF droplet and incubated for 4 h at 37°C. After co-incubation, signs of successful fertilization

(extrusion of the second polar body and / or pronucleus formation) were recorded and zygotes were washed in HTF and cultured until collection at 24 h (two-cell stage). At collection, two-cell embryos were washed of HTF media in PBS containing 3.0 mg/mL polyvinylpyrrolidone (PBS/PVP) before a brief incubation in Acid Tyrode's solution to remove the zona pellucida (ZP). ZP-free embryos were carefully washed several times, pooled and prepared for RNA extraction.

### **RNA extraction and sequencing of two-cell embryos and bioinformatic analysis**

Total RNA was extracted from three groups of two-cell embryos, including those fertilized by spermatozoa isolated from control, AA-E or AA-S mice using a Direct-zol RNA MicroPrep Kit (Zymo Research Corporation) according to the manufacturer's instructions. This preparation of total RNA was subjected to Nugen trio kit RNA sample kit preparation as per the manufacturer's instructions at BGI (BGI, Shenzhen, China). Each library was sequenced in triplicate using a DNBseq platform. Raw sequence reads were filtered using BGI's internal software SOAPnuke (v1.5.2), to remove reads with adaptors, unknown bases greater than 0.1% and low quality reads (defined as those with more than 20% of bases with a quality lower than 10). Remaining clean reads were mapped against the *Mus musculus* genome (mm10) using HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts v2.0.4). Processed sequenced reads were imported into DEseq2 to assess differential mRNA expression between treatment groups. Differential expression was calculated for each individual gene using a negative binomial distribution as previously described<sup>84</sup>. Transcriptome lists were refined to identify unique genes for each respective group. Such refining involved retaining genes for each individual experimental group that were identified across all three replicates and had an average FPKM (Fragments Per Kilobase of transcript per Million mapped reads)  $\geq 1$  across these replications.

### **Reverse transcription and quantitative real-time PCR of selected small RNA candidates**

Validation of differentially accumulated sncRNAs was conducted using a quantitative real-time PCR (RT-qPCR) strategy with Taqman pre-designed miRNA and custom small RNA assays, according to the manufacturer's instructions. RNA isolated from three separate biological replicates distinct to those used for sncRNA sequencing was utilized. Pre-designed miRNA assays for *miR-30a-5p* (assay ID. 000417), *miR-30b-5p* (assay ID. 000602), *miR-20a-5p* (assay ID. 000580), *miR-139-5p* (assay ID. 002289), *miR-152-3p* (assay ID. 000475) and *miR-let-7d* (assay ID: 001178) and custom small RNA assays for *piR-215* and *piR-123453* were utilized. Quantitative RT-PCR was performed on cDNA generated from 1.0 µg of total RNA using a Light Cycler 96 SW 1.1 (Roche, Castle Hill, Australia). The U6 small nuclear RNA (assay ID. 001973) or *miR-let-7b* (assay ID. 002619) was employed as endogenous controls to normalize the expression levels of target miRNAs, and relative expression levels were calculated using the  $2^{-\Delta Ct}$  and  $2^{-\Delta\Delta Ct}$  method, where appropriate<sup>85</sup>.

### ***In silico* analysis of sequencing and proteomic datasets**

*In silico* analysis of cauda spermatozoa sncRNA sequencing, epithelial cell proteome and 2-cell embryo transcriptome data sets was undertaken using a suite of techniques. Briefly, abundance / accumulation data were assessed via volcano plots to visualize trends associated with differentially expressed sncRNA / genes / proteins associated with acrylamide exposure. Hierarchical clustering was performed using the Perseus software suite version (1.6.10.43)<sup>86</sup> to generate heat maps. Datasets were also interrogated using Ingenuity® Pathway Analysis (IPA) software (Qiagen, Hilden, Germany) for enrichment of functional pathways and to predict the molecular networks that DEGs were known to function in. IPA was also utilized to generate a list of mRNA targets of the miRNAs that were differentially abundant in the sperm from acrylamide exposed mice. This analysis was restricted to those mRNA targets that had been experimentally validated for each miRNA. The identification of *MIR* genes regulated by acrylamide responsive transcription factors was facilitated by the literature-curated database of experimentally validated TF-miRNA regulations, TransmiR (v2.0)<sup>39</sup>.

## **Statistical analysis**

Data presented in this manuscript are expressed as mean values  $\pm$  standard error. Statistical analyses were performed using JMP software (version 14.2.0; SAS Institute INC, Cary, NC, USA), using unpaired Student's *t*-tests to determine statistical significance.  $P \leq 0.05$  was considered significant, with the level of significance denoted by asterisks such that  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*) and  $P \leq 0.001$  (\*\*\*)<sup>†</sup>. Experiments were performed at minimum in triplicate unless otherwise stated.

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## **Author Contributions**

NAT, BN, MDD, SDR, ALE and GND conceived and designed the experiments. NAT carried out the experiments with technical assistance from WZ, ALA and SJS. DAS-B assisted with mass spectrometry experiments and analysis. MJX performed bioinformatic analysis on the sperm sncRNA sequencing data. NAT and BN generated the manuscript draft and all co-authors edited the manuscript and provided their approval of the final version.

## **Competing Interests statement**

The authors declare no competing interests

## **Data Availability**

The data discussed in this publication have been deposited in the respective repositories. RNA-seq and ncRNA-seq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession numbers GSE162527 with the accession

token gjwlgkkivzifxkf. Mass spectrometry data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE repository<sup>87</sup> for discovery TMT data with the dataset identifier PXD022865 and 10.6019/PXD022866. Reviewer account details: Username: reviewer [pxd022865@ebi.ac.uk](mailto:pxd022865@ebi.ac.uk) Password: hG8C9Pc or via Panorama Public<sup>88</sup> (<https://panoramaweb.org/yjOG1.url>) for targeted PRM data with the data set identifier PXD022876. Reviewer account details: Username: [panorama+nixon@proteinms.net](mailto:panorama+nixon@proteinms.net) Password: VzrBixow.

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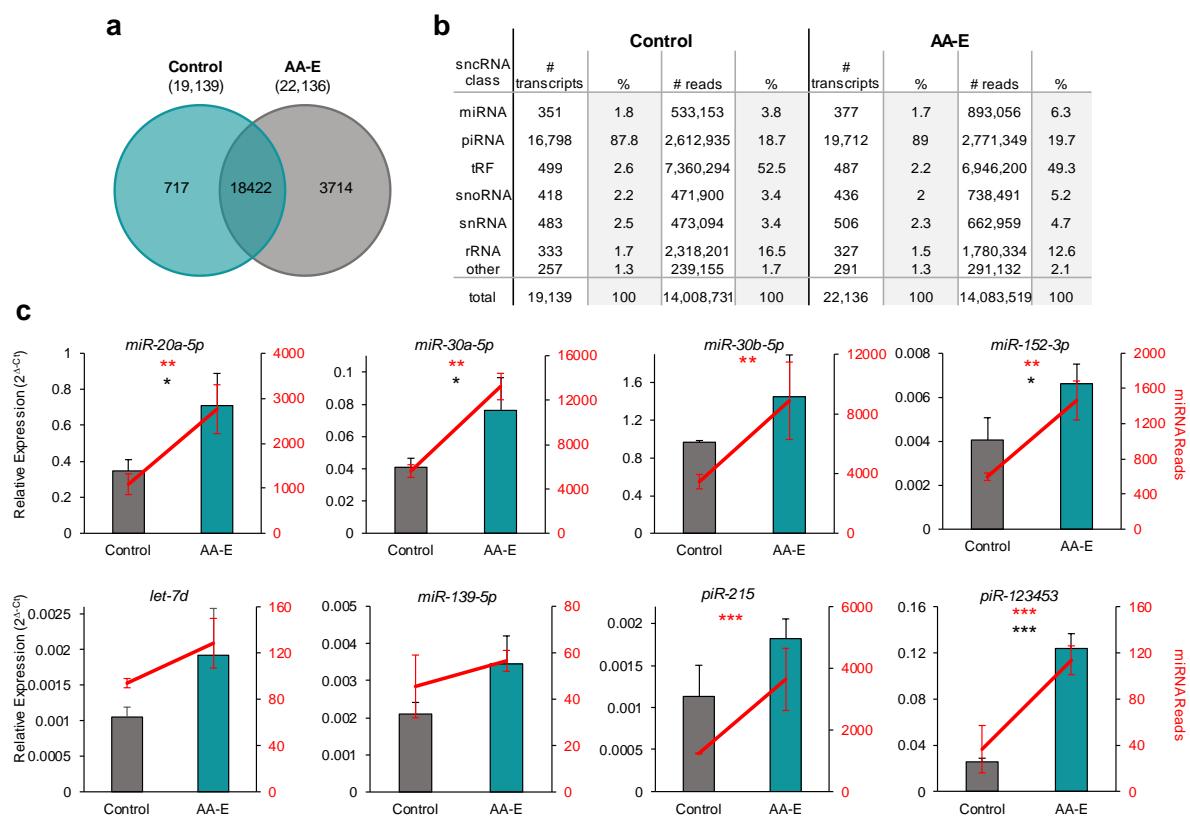
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## Supplementary Figure 1



**Supplementary Figure 1:** **a** Venn diagram of sncRNA transcripts in cauda spermatozoa of control and epididymal acrylamide exposed mice (AA-E), demonstrating a large proportion of sncRNAs are common to both groups, with a greater number of unique (only present in one sample, with a read cut off for presence > 10 reads) transcripts in acrylamide group compared to control. **b** Average number of transcripts and corresponding number of reads and percentage for each sncRNA class contributing to the overall sncRNA landscape of control and AA-E cauda spermatozoa. Column 4 (% reads) data used to produced Fig 2A. **c** Experimental validation of differentially accumulated miRNA and piRNA sncRNAs. To validate next generation sequencing data, six candidates displaying significantly different levels of accumulation and two candidates that remained unchanged in spermatozoa exposed to acrylamide were selected for targeted validation using RT-qPCR. These experiments were performed in triplicate using 3-5 distinct pools of biological samples ( $n = 3-4$  mice per sample) differing to those employed for next generation sequencing analyses. The U6 small nuclear RNA was employed as an endogenous control to normalise the expression levels of target sncRNAs. Data are presented as mean  $\pm$  SEM. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .

**Supplementary Table 1:** Summary of mouse caput epididymal epithelial cell proteome data set.

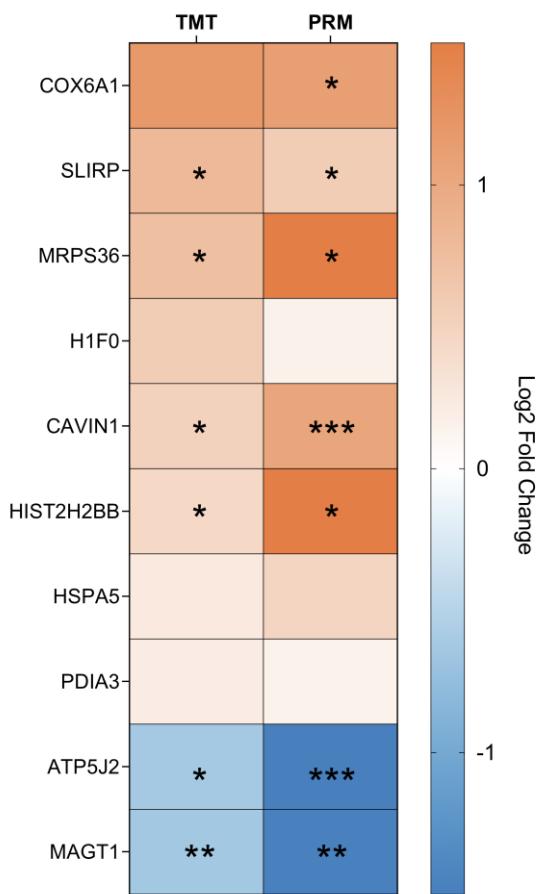
Total proteins identified	Av. Peptide hits/protein	Av. Unique peptide hits/protein	Av. Protein coverage (%)	Number of differentially accumulated proteins (fold change $\pm 1.5$ )	
Mouse caput epididymal epithelial cells	4,405	12.1	10.8	26.9	302

**Supplementary Table 2: Alternate pathways for sperm miRNA change following paternal exposure to acrylamide.** Proteins associated with miRNA biogenesis, packaging of miRNA into extracellular vesicles (EVs) or EV biogenesis (ESCRT: the endosomal sorting complexes required for transport) and identified in proteomic analysis of caput epididymal epithelial cells exposed to acrylamide. Bold ratio indicates *P*-value ≤0.05

Function	Protein ID	Gene symbol	Accession	Abundance Ratio (Acrylamide / Control)	References
DNA methylation	Methyl-CpG-binding protein 2	Mecp2	Q9Z2D6	1.043	As reviewed in <sup>1,2,3,4</sup>
	DNA (cytosine-5)-methyltransferase 1	Dnmt1	P13864	1.015	
	Histone deacetylase 2	HDAC2	P70288	0.896	
	Histone deacetylase 6	HDAC6	Q9Z2V5	<b>0.821</b>	
	Histone deacetylase 1	HDAC1	O09106	0.82	
RNA biogenesis	Exportin-5	Xpo5	Q924C1	<b>0.64</b>	As reviewed in <sup>5</sup>
ESCRT	Signal transducing adapter molecule 1	Stam	P70297	<b>1.623</b>	As reviewed in <sup>6,7</sup>
	Tumor susceptibility gene 101 protein	Tsg101	Q61187	<b>0.59</b>	
	Vacuolar protein sorting-associated protein 37B	Vps37b	Q8R0J7	1.029	
	Vacuolar protein sorting-associated protein 37C	Vps37c	Q8R105	1.153	
	Vacuolar protein sorting-associated protein 28 homolog	Vps28	Q9D1C8	0.73	
	Multivesicular body subunit 12A	Mvb12a	Q78HU3	1.118	
	Vacuolar protein-sorting-associated protein 36	Vps36	Q91XD6	0.969	
	Charged multivesicular body protein 6	Chmp6	P0C0A3	2.414	
	Charged multivesicular body protein 4b	Chmp4b	Q9D8B3	1.136	
	Charged multivesicular body protein 3	Chmp3	Q9CQ10	<b>0.81</b>	
	Charged multivesicular body protein 2a	Chmp2a	Q9DB34	0.911	
	Charged multivesicular body protein 2b	Chmp2b	Q8BJF9	1.289	
	Charged multivesicular body protein 1a	Chmp1a	Q921W0	1.018	
	Charged multivesicular body protein 1b-1	Chmp1b	Q99LU0	0.998	

	Charged multivesicular body protein 5	Chmp5	Q9D7S9	<b>0.766</b>	
	Charged multivesicular body protein 7	Chmp7	Q8R1T1	0.779	
	IST1 homolog	Ist1	Q9CX00	0.794	
	Vacuolar protein sorting-associated protein 4A	Vps4a	Q8VEJ9	0.856	
	Vacuolar protein sorting-associated protein 4B	Vps4b	P46467	0.703	
	Vacuolar protein sorting-associated protein VTA1 homolog	Vta1	Q9CR26	<b>1.092</b>	
	Ubiquitin-60S ribosomal protein L40	Uba52	P62984	0.874	
	Ubiquitin-40S ribosomal protein S27a	Rps27a	P62983	0.711	
miRNA packaging into EV	Heterogeneous nuclear ribonucleoproteins A2/B1	Hnrnpa2b1	O88569	0.964	
	Heterogeneous nuclear ribonucleoprotein A1	Hnrnpa1	P49312	1.148	
	Heterogeneous nuclear ribonucleoprotein U	Hnrnpu	Q8VEK3	0.977	
	Isoform 2 of Heterogeneous nuclear ribonucleoprotein Q	Syncrip	Q7TMK9-2	1.058	
	Y-box-binding protein 1	Ybx1	P62960	0.976	
	Major vault protein	Mvp	Q9EQK5	0.974	
	Isoform 2B of GTPase KRas	Kras	P32883-2	0.923	
	Argonaute 2	Ago2	Q8CJG0	<b>1.154</b>	
	Heat shock protein HSP 90-beta	Hsp90ab1	P11499	0.638	
	Heterogeneous nuclear ribonucleoprotein M	Hnrnpm	Q9D0E1	<b>1.184</b>	

As reviewed in<sup>8,9</sup>

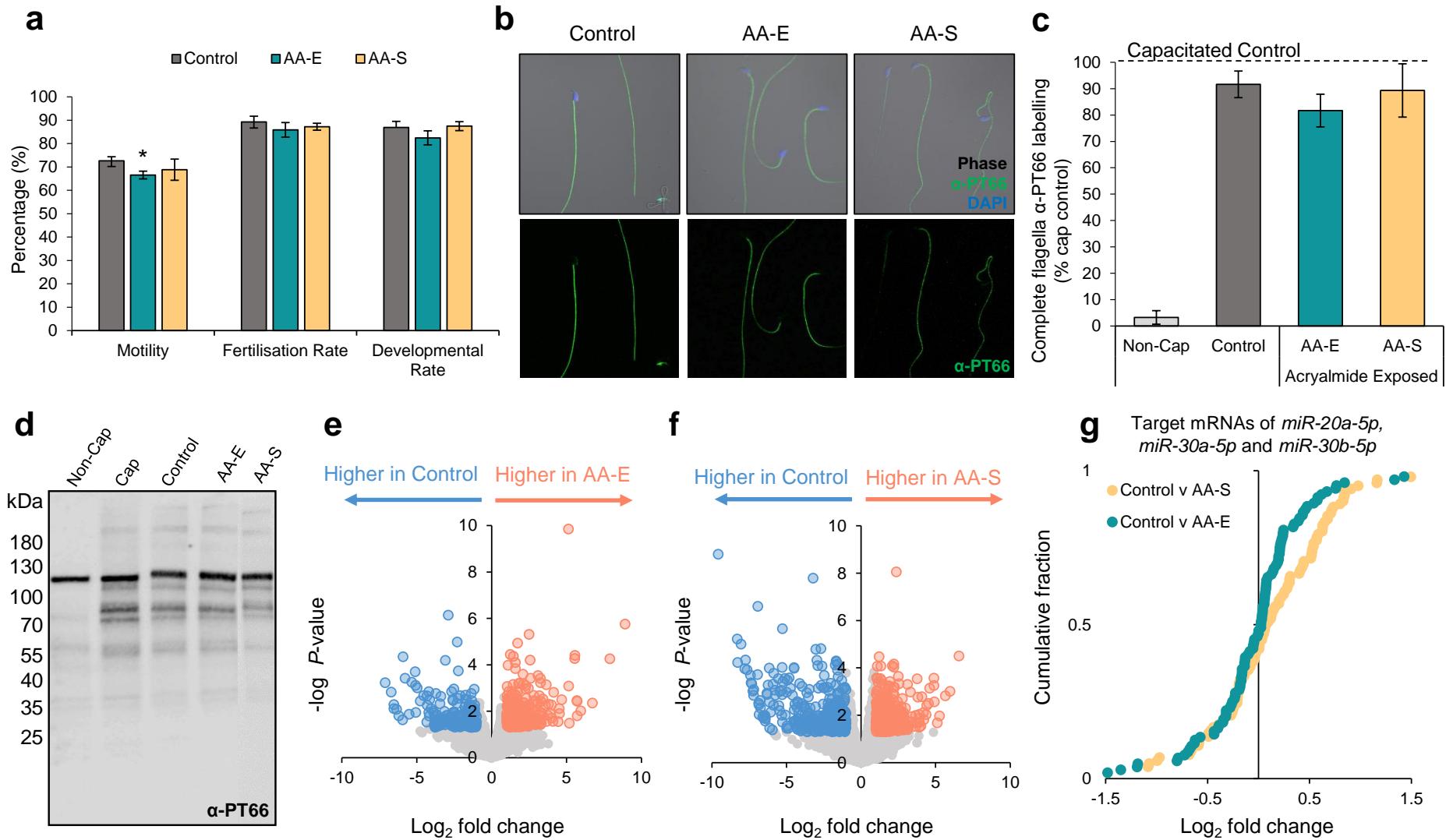


**Supplementary Figure 2: Validation of caput epididymal epithelial cell protein abundance.** Using an independent targeted proteomics approach (parallel reaction monitoring; PRM), ten highly abundant candidates were selected. Six candidates displaying significantly altered abundance and four candidates that remained unchanged in epididymal epithelial cells following acrylamide exposure. Data is displayed as a heatmap depicting  $\log_2$  fold change between control and acrylamide exposed caput epididymal epithelial cells identified via quantitative approach (TMT) and targeted approach (PRM). These experiments were performed in triplicate. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .

**Supplementary Table 3: Putative transcription factor binding sites.** Underlined transcription factor name depicts those represented in Fig. 4f.

		Site (5'- 3')		
	Transcription factor	Start (nt)	End (nt)	Sequence
Chr 1: <i>Mir30a</i>	<u>NR3C1</u>	-2120	-2112	<b>AGAACAGA</b>
	<u>RELA</u>	-1608	-1601	<b>GGAATT</b>
	<u>NR3C1</u>	-96	-86	<b>ACAGAATGTT</b>
Chr 11: <i>Mir152</i>	<u>NR3C1</u>	-2078	-2071	<b>AGAACAG</b>
	<u>NR3C1</u>	-804	-797	<b>AGAACAG</b>
	<u>CTCF</u>	-435	-427	<b>GGGGCGC</b>
	<u>NR3C1</u>	-70	-61	<b>CAGAATGAT</b>
Chr 14: <i>Mir18/Mir20</i>	<u>NR3C1</u>	-4883	-4876	<b>GAACAGA</b>
	<u>RELA</u>	-3636	-3627	<b>GGAATTCC</b>
	<u>CTCF</u>	-3056	-3041	<b>GCCACCAGCGGCTCC</b>
	<u>CTCF</u>	-2268	-2252	<b>GGCCGGGAGGGGGCGC</b>
	<u>NR3C1</u>	-280	-271	<b>ACAGAATT</b>

**Supplementary Figure 3**



**Supplementary Figure 3: Acute acrylamide exposure does not influence spermatozoa functionality or fertilising competency.** Adult male mice were administered either acrylamide (25 mg/kg w/day) or control via intraperitoneal injection for five consecutive days. Mature spermatozoa were collected from mice sacrificed three or twenty-four days following the last injection, such that the isolated sperm were exposed to acrylamide as spermatozoa in the epididymis (AA-E) or as spermatocytes in the testis (AA-S). Spermatozoa were swam out in capacitation media under mineral oil in preparation for *in vitro* fertilisation (IVF) for 45 min. **a** Following incubation total motility was recorded by counting 100 cells using light microscopy. Fertilisation rate was determined for each group as a percentage of fertilised oocytes over the total number of viable oocytes collected. Presumptive zygotes were cultured for 24 h until 2-cell development and development rate was scored as the number of 2-cell embryos over number of fertilised oocytes. **b** Sperm capacitation competency was assessed by immunoblotting (B) and immunocytochemistry **c** with anti-phosphotyrosine antibodies ( $\alpha$ -PT66). The experiment was repeated three times with the addition of a non-capacitated (Non-Cap) and capacitated (Cap) control. Representative images for immunocytochemical analysis were captured using a confocal microscope. **d** The percentage of cells displaying ‘complete’ phosphotyrosine labelling (fluorescence observed over the entire flagella) was determined by recording fluorescence staining pattern of 100 cells per replicate. Data presented represents mean  $\pm$  SEM. \* $P<0.05$  **e, f** Volcano plots depicting  $\log_2$  fold change (x-axis) and  $-\log P$ -value (y-axis) of genes shared between control and AA-E or AA-S embryo, respectively. Thresholds of fold change  $\pm 2$  and  $P$ -value  $\leq 0.05$  were assigned to determine differentially expressed genes. **G** Cumulative distribution plot depicting expression differences for miRNA target genes of the three validated miRNAs in AA-E and AA-S embryos compared to control.

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## CHAPTER THREE: ORIGINAL RESEARCH ARTICLE

# A novel role for milk fat globule-EGF factor 8 protein (MFGE8) in the mediation of sperm-extracellular vesicle interactions

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## CHAPTER OVERVIEW:

Due to the highly specialised testicular differentiation process that culminates in the production of morphologically mature spermatozoa, these cells are rendered transcriptionally and translationally silent. Accordingly, spermatozoa rely on the epididymal extracellular environment to support their functional maturation and viability. Indeed, spermatozoa undergo dramatic remodelling of their macromolecular cargo, facilitated by the secretions of the epididymal soma. As established throughout this thesis (Chapters 1 and 2), an important constituent of the epididymal extracellular environment are epididymosomes. These extracellular vesicles have emerged as key players in modification of the macromolecular landscape of transiting epididymal spermatozoa. Further, we and others have demonstrated the role of epididymosomes in mediating a molecular response to prevailing paternal environmental conditions (Chapter 2), thus highlighting the integral role these vesicles hold. Accordingly, a greater understanding of the molecular mechanisms that facilitate epididymosome-sperm interactions holds promise in terms of alleviating pathological responses to paternal insult and prevention of altered sperm sncRNA profiles. While little is known of the mechanistic basis by which epididymosomes deliver their cargo, ongoing research from within our laboratory has developed a model whereby interaction first involves an adhesion event followed by transient fusion and dispersal of cargo.

The aims of the studies reported in this chapter were to characterise extracellular vesicles (EVs) released by an immortalised mouse caput epididymal epithelial cell line (mECap18), assess their ability to interact and deliver cargo to isolated mouse spermatozoa *in vitro* and exploit this *in vitro* cell line to further understand the mechanisms of EV-sperm interaction. Data presented within this chapter demonstrate that mECap18 cells produce EVs *in vitro* that closely resemble native epididymosomes. Additionally, the protein composition of mECap18 EVs resembled epididymosomes and co-incubation experiments revealed these vesicles also share functional characteristics, delivering protein and RNA cargo to caput spermatozoa. Guided by previous preliminary studies implicating milk fat globule-EGF factor 8 protein (MFGE8) in sperm-epididymosome interaction we focused on this protein ligand and built evidence to substantiate its role in facilitating the efficient exchange of EV cargo to mouse spermatozoa.

Manuscript accepted to *Proteomics* with minor revisions

27-Nov-2020

Dear Dr. Nixon,

Your manuscript pmic.202000079 "A novel role for milk fat globule-EGF factor 8 protein (MFGE8) in the mediation of sperm-extracellular vesicle interactions" submitted to PROTEOMICS has been reviewed. I am pleased to inform you that the reviewers have recommended **acceptance of your paper after a minor revision**. The comments of the referees and editors are included at the bottom of this letter.

Before you submit your revision, please carefully proof-read the manuscript to minimize typographical, grammatical, and bibliographic errors. In addition, please check to make sure that all abbreviations are defined.

You can upload your revised manuscript and submit it through your Author Center.

Once again, thank you for submitting your work to PROTEOMICS. I look forward to receiving your revision.

Sincerely,

Dr. Lucie Kalvodova  
PROTEOMICS Editor

Please note the manuscript included in this thesis comprises the revised version of the submitted manuscript addressing the reviewer responses.

**Title: A novel role for milk fat globule-EGF factor 8 protein (MFGE8) in the mediation of mouse sperm-extracellular vesicle interactions**

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**Short title:** MFGE8 mediates sperm-extracellular vesicle interaction

**Keywords:** epididymis, epididymosome, extracellular vesicle, milk fat globule-EGF factor 8 protein (MFGE8), spermatozoa, sperm maturation

## **ABSTRACT**

Spermatozoa transition to functional maturity as they are conveyed through the epididymis, a highly specialized region of the male excurrent duct system. Owing to their transcriptionally and translationally inert state, this transformation into fertilization competent cells is driven by complex mechanisms of intercellular communication with the secretory epithelium that delineates the epididymal tubule. Chief among these mechanisms are the release of extracellular vesicles (EV), which have been implicated in the exchange of varied macromolecular cargo with spermatozoa. Here, we describe the optimization of a tractable cell culture model to study the mechanistic basis of sperm-extracellular vesicle interactions. In tandem with receptor inhibition strategies, our data demonstrate the importance of Milk Fat Globule-EGF Factor 8 (MFGE8) protein in mediating the efficient exchange of macromolecular EV cargo with mouse spermatozoa; with the MFGE8 integrin-binding Arg-Gly-Asp (RGD) tripeptide motif identified as being of particular importance. Specifically, complementary strategies involving MFGE8 RGD domain ablation, competitive RGD-peptide inhibition and antibody-masking of alpha V integrin receptors, all significantly inhibited the uptake and redistribution of EV-delivered proteins into immature mouse spermatozoa. These collective data implicate the MFGE8 ligand and its cognate integrin receptor in the mediation of the EV interactions that underpin sperm maturation.

## INTRODUCTION

Successful fertilization is underpinned by the union of gametes, with each cell having first attained the necessary level of functional maturity [1]. In the case of the spermatozoon, this journey to maturity is initiated within the testes during which time the germline undergoes sequential mitotic and meiotic divisions followed by cytoplasmic and nuclear remodeling to eventually give rise to one of the most highly differentiated cells in the mammalian body [2]. Notwithstanding this morphological transformation, mammalian sperm cells only acquire the acumen to fertilize an ovum during their carriage through the epididymal portion of the male extragonadal duct system [3]. This conversion into fertilization competent cells is accompanied by substantive modification of the sperm proteomic [4] and epigenetic landscapes [5]; processes that occur in the complete absence of nuclear gene transcription and protein translation. Instead, mammalian sperm maturation is driven exclusively by the interplay of sophisticated forms of intercellular communication with the epididymal soma [6]. Chief among these mechanisms is the secretion of extracellular vesicles (EVs), termed epididymosomes, which have been implicated in the exchange of varied forms of macromolecular cargo with spermatozoa [7-10].

Epididymosomes originate within the epithelial cells of the mammalian epididymis as constituents of multivesicular bodies [9], before being shed into the lumen of the duct whereupon they interact with spermatozoa and downstream epithelial cells [11, 12]. The extent to which epididymosomes influence their recipient cells is highlighted by recent compositional analyses, which have revealed these EVs encapsulate a diverse cargo of proteins [13, 14], small non-coding RNAs [7, 11, 15], and lipids [8, 16]. Moreover, the development of tractable *in vitro* co-culture assays has confirmed the transfer of each of these forms of cargo between epididymosomes and spermatozoa [7, 10]. Such assays have also begun to yield important insights into the mechanisms by which epididymosome cargo is exchanged, revealing the important influences of temperature, pH and ionic concentration [9]. Similarly, a combination of pharmacological intervention and differential labeling strategies has provided evidence that

mouse epididymosome-sperm interactions can be resolved into two sequential phases involving rapid tethering followed by a transient membrane fusion and cargo delivery [10]. Despite these advances, we remain uncertain about the full complement of cognate receptor – ligand interactions that mediate the tethering of epididymosomes to the sperm head as well as the signaling pathway(s) that are engaged in response to this interaction. With the intention of bridging this important knowledge gap, here we explore the role of Milk Fat Globule-EGF Factor 8 (MFGE8) protein in the mediation of mouse sperm-EV interactions.

In guiding the target selection for this study, our previous work has demonstrated that antibody masking of epididymosome-resident MFGE8 effectively reduces the ability of these vesicles to bind mouse spermatozoa (Nixon et al., 2019b). This finding builds on evidence that MFGE8 acts as an adhesion molecule responsible for the orchestration of diverse cellular interactions [17]. Such activity has been linked to the presence of an RGD integrin-binding motif, which is capable of engaging  $\alpha\beta3/5$  integrin heterodimers to facilitate cell adhesion and induce integrin-mediated signal transduction [17]. In terms of the male reproductive tract, MFGE8 was originally characterized as a sperm protein with binding affinity for glycoproteins of the egg coat [18]; a function that is conserved in both porcine and mice [19]. It follows that male mice harboring an *Mfge8*-null mutation are sub-fertile, producing spermatozoa that are unable to bind eggs *in vitro* [19]. Notably, mouse sperm first acquire MFGE8 within the Golgi complex of spermatogenic cells, however, the majority of sperm-associated MFGE8 is derived from secretions of the proximal epididymis. Here, we offer mechanistic insight into the mode of MFGE8 transfer to maturing spermatozoa via EVs, and also propose a functional role for the MFGE8 RGD domain in the engagement of  $\alpha V$  integrin receptors on spermatozoa.

## MATERIALS AND METHODS

### Reagents

All reagents were of molecular biology or research grade and unless specified, were obtained from Merck (Burlington, MA, USA) or Thermo Fisher Scientific (Waltham, MA, USA). A summary of all antibodies used this study is provided in Supplementary Table S1.

### Ethics Statement

All experimental procedures involving animals were conducted with the approval of the University of Newcastle's Animal Care and Ethics Committee (approval numbers A-2013-322 and A-2018-826), in accordance with relevant national and international guidelines. Swiss mice were housed under a controlled lighting regime (12L: 12D) at 21–22°C and supplied with food and water *ad libitum*. Prior to dissection, animals were euthanized via CO<sub>2</sub> inhalation.

### mECap18 cell culture and extracellular vesicle isolation

An immortalized mouse caput epididymal (mECap18) cell line was utilized to harvest EVs under normal *in vitro* incubation conditions <sup>[20]</sup> and in cells subjected to CRISPR-Cas9 genome editing to generate mutations in the target gene, *Mfge8* (see below). Cells were grown to ~70% confluence, washed three times with sterile phosphate buffered saline (PBS) and incubated in serum free media for 24 h before the collection of conditioned media for EV isolation.

Conditioned media (15 mL) was carefully aspirated and cleared of cellular debris by two sequential centrifugation steps at 500 × g and 2000 × g for 5 min each at 4°C. The resultant supernatant was concentrated by centrifugation using an Amicon Ultra-15 Centrifugal Filter Unit (30 kDa cut-off; Merck) at 3000 × g at 4°C to a volume of ~200 – 400 µL prior to further centrifugation steps at increasing velocity (4000 × g, 8000 × g and 17,000 × g for 15 min at 4°C per centrifugation) to eliminate all traces of cellular debris. The supernatant (~450 µL) was gently overlaid upon a discontinuous iodixanol-based density gradient (comprising 40%, 20%, 10% and 5% suspensions) (OptiPrep; Merck) before being centrifuged at 100,000 × g for 18 h at 4°C as previously described <sup>[7]</sup>. Thereafter, twelve equal fractions (185 µL / fraction)

were recovered, diluted in PBS, and subjected to a final ultracentrifugation step at 100,000 × g for 3 h at 4°C. The resulting pellets were processed as described below.

#### **mECap18 extracellular vesicle characterization**

Initial characterization of fractionated samples focused on confirming the presence of recognized EV markers (FLOT1, CD9, CD63) by immunoblotting according to [7]. The identification of EV markers predominantly within fractions 9 and 10, prompted the pooling of these fractions for all subsequent experiments. EVs were characterized based on mean particle size and heterogeneity via analysis on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) [7]. EV morphology, purity and size were also assessed by conventional transmission electron microscopy [7, 21] and by concentration onto aldehyde/sulfate latex beads followed by sequential labeling with anti-FLOT1 and Alexa Fluor 488-conjugated secondary antibodies [7]. Finally, EVs and mECap18 cell lysates were prepared for immunoblotting with a suite of antibodies (CD63, CD9, PSMD7, FLOT1, HSP90B1, APOA1, GAPDH) recommended for EV validation [22].

#### **Proteomic comparison of mECap18 extracellular vesicles and mouse caput epididymosomes**

To examine the conservation of the physical characteristics of mECap18 EVs and that of native epididymosomes, the latter were isolated from the caput epididymal tissue of adult male mice as previously described [7, 10]. After isolation, mECap18 EVs and epididymosomes were lysed separately (7 M urea, 2 M thiourea, 4% (w/v) CHAPS for 1 h on ice with regular vortexing) and 40 µg of protein from each vesicle lysate was labeled with 320 pmol of appropriate cyanine-dye reagents (i.e. either Cyanine3 or Cyanine5 esters) for 1 h on ice. Labeling reactions were quenched by addition of excess L-lysine (10 mM, 10 min on ice) after which the differentially labeled samples were combined, resolved by 2D SDS-PAGE and imaged using a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA) [14].

#### **Transfer of extracellular vesicle protein and microRNA cargo to mouse spermatozoa**

The interaction of mECap18 EVs with spermatozoa was assessed by biotinylation of their proteomic contents (membrane impermeable: EZ-Link sulfo-NHS-LC-Biotin; membrane permeable: EZ-Link BMCC-Biotin; Thermo Fisher Scientific) as previously described [10]. Labeled EVs were subsequently co-incubated with caput epididymal sperm for 1 or 3 h at 37°C in an atmosphere of 5% CO<sub>2</sub> [10], with visualization of the sperm domains harboring transferred biotinylated cargo being achieved via affinity labeling with Alexa Fluor 488 conjugated streptavidin. Images were captured by confocal microscopy (Olympus FV1000; Olympus, Shinjuku, Tokyo, Japan) and transfer efficiency was assessed by counting the percentage of labeled spermatozoa. Controls to discriminate the specificity of EV mediated protein transfer included naïve populations of unlabeled spermatozoa, sperm labeled directly with biotin, and sperm incubated with unlabeled EVs.

Using an identical co-incubation strategy, we also investigated the exchange of microRNA (miRNA) cargo between EVs and recipient spermatozoa. Following incubation, sperm cells were processed for total RNA extraction as previously described [7]. The abundance of candidate miRNAs [*miR-191-5p* (assay ID 002299), *miR-375-3p* (assay ID 000564), *miR-467a-5p* (assay ID 001826) and *miR-467e-5p* (assay ID 002568)] was examined by quantitative real-time PCR (RT-qPCR) with TaqMan miRNA assay reagents in accordance with the manufacturer's instructions (Thermo Fisher Scientific). RT-qPCR data was normalized against the U6 small nuclear RNA (snRNA; assay ID 001973) and relative abundance was calculated using the 2<sup>-ΔCt</sup> method [23].

#### **Generation of CRISPR-Cas9-mediated *Mfge8* mutant mECap18 cells**

All CRISPR-Cas9 reagents were obtained from Thermo Fisher Scientific. Prior to transfection, mECap18 cells were seeded at 150,000 cells/well into 12-well plates without antibiotics. Cells were then transfected with 1,250 ng TrueCut Cas9 Protein v2, 315 ng TrueGuide gRNA (A35510; ID: CRISPR475261\_SG), 2.5 µL lipofectamine Cas9 with PLUS solution and 4.0 µL lipofectamine 3,000 as per manufacturer's instructions (Thermo Fisher Scientific). An internal transfection control (mCherry Red) was used to estimate transfection efficiency. Cells were

allowed to recover for 24 h at 37°C in 5% CO<sub>2</sub> before being re-seeded into 6-well plates and cultured for 72 h. Transfected cells were harvested and divided for genome editing quantification using a GeneArt Genomic Cleavage Detection Kit (A24372; Thermo Fisher Scientific) with *Mfge8* flanking primers (5' - CTGGTCTTGGCTCCAAGT - 3' and 5' - ATGTGGGCAACTGATCC - 3') as per manufacturer's instructions, and the resultant cell population was then replated as single cells in 96-well plates using limited dilution cloning. Resulting colonies were expanded and gDNA Sanger sequencing was performed to detect deletions.

#### ***Mfge8* mutant cDNA transcript validation**

One wild-type and two mutant lines were selected for analysis based on their gDNA sequences and used to characterize the specifics of CRISPR-Cas9-induced deletions. Cells from these lines were harvested and total cellular RNA (3.0 µg) was reverse transcribed using 500 ng Oligo(dT)15 primer, 5x buffer, 100 mM dithiothreitol, 40 U RNasin ribonuclease, 0.5 mM dNTPs and 20 Units (U) of Moloney murine leukemia virus reverse transcriptase. Subsequent PCR reactions were performed with the equivalent of 100 ng of RNA, 80 ng of each primer (5' - GCATGCTACTCTGCGCCTC - 3' and 5' – GCTGCTGGGCTGTTAATGCTC - 3'), 0.5 mM dNTPs, 1 U of GoTaq DNA Polymerase (Promega, Madison, WI) and 1.5 mM MgCl<sub>2</sub>. DNA from PCR products was purified from 1.0% (w/v) agarose gels using a Wizard SV Gel and PCR Clean-Up System (Promega) and cloned into the pGEM-T Easy Vector as per the manufacturer's instructions (Promega). Extracted plasmid DNA was subjected to Sanger sequencing.

#### **Inhibition of extracellular vesicle cargo transfer to mouse spermatozoa**

Three complementary strategies were assessed for their ability to inhibit protein transport from EVs to mouse spermatozoa. We first determined whether the EVs generated by *Mfge8* mutant mECap18 cell lines were refractory to sperm interaction. For this purpose, EVs were isolated from each of three cell lines (one wild-type and two *Mfge8* mutant lines) before being labeled

with biotinylation reagents and co-incubated with caput epididymal spermatozoa as described above. Secondly, we assessed the impact of competitive RGD-peptide mediated inhibition of EV-sperm interactions, whereby caput sperm were incubated with either 200 µM RGD tripeptide, 200 µM RAD control tripeptide (SCP0157 and SCP0156, respectively; Merck) or a PBS vehicle control. Incubations were conducted for 30 min at 37°C before the spermatozoa were washed and exposed to biotin labeled EVs. Co-incubation was continued for either 1 or 3 h at 37°C in 5% CO<sub>2</sub>, after which spermatozoa were washed to remove unbound or peripherally adherent EVs and then fixed in 4% paraformaldehyde. Domains harboring transferred biotinylated proteins were detected using Alexa Fluor 488 conjugated streptavidin and confocal microscopy, and the percentage of labeled sperm recorded. The final strategy focused on the ability of antibody-mediated masking of the integrin alpha V receptor to perturb sperm-EV interactions. For this purpose, caput sperm were incubated in either anti-integrin alpha V antibodies (100 µg/mL), of the equivalent isotype-matched IgG (100 µg/mL) or a PBS vehicle control. After 40 min incubation at 37°C, spermatozoa were washed and exposed to biotin labeled EVs. Co-incubation was continued for either 1 or 3 h at 37°C in 5% CO<sub>2</sub>, after which the sperm cells were washed, fixed and assessed for biotin transfer.

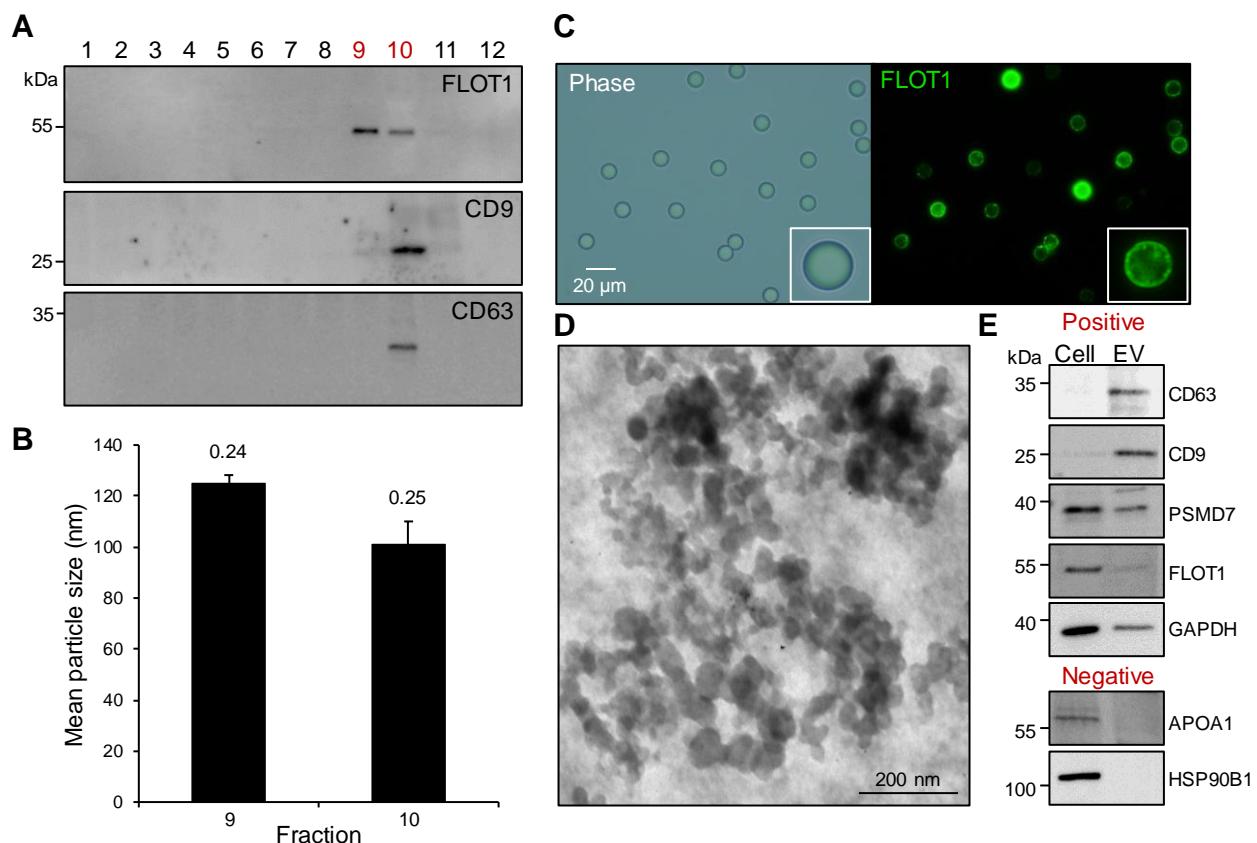
### **Statistical analyses**

All experiments were replicated a minimum of three times, with pooled samples of spermatozoa and epididymosomes having been obtained from at least three male mice. For the purpose of assessing biotin labeling profiles, ≥ 100 spermatozoa were counted in each sample through blind assessment and the corresponding percentage of cells with post-acrosomal domain or whole head labeling was determined. Graphical data are presented as mean values ± SEM. Statistical significance was determined using either a one-way ANOVA or two-way ANOVA with Tukey or Šidák correction for the appropriate multiple comparisons. Analysis was performed on GraphPad Prism version 8.4.3 for Windows (GraphPad Software, San Diego, CA).

## RESULTS

### The mECap18 cell line secretes an abundance of extracellular vesicles *in vitro*

Initial experiments focused on optimization of an immortalized mouse caput epididymal (mECap18) cell line as a model with which to study the mechanisms of EV-sperm interactions. Density gradient centrifugation of conditioned media harvested from cultured mECap18 cells yielded enriched populations of EVs (Fig. 1A; fractions 9 and 10) with a buoyant density equivalent to that of native epididymosomes [7]. These fractions tested positive for EV markers (FLOT1, CD9, CD63) by immunoblotting, and sizing analysis revealed they contained a relatively homogenous population (polydispersity indices of 0.24 and 0.25) of EVs of ~100 to 130 nm (Fig. 1B). Accordingly, fractions 9 and 10 were pooled and subjected to an additional suite of assays to confirm they contained EVs with characteristics that satisfied MISEV2018 guidelines [22]. Specifically, EVs were concentrated via adherence to aldehyde/sulfate latex beads enabling them to be visualized via labeling with anti-FLOT1 antibodies (Fig. 1C).

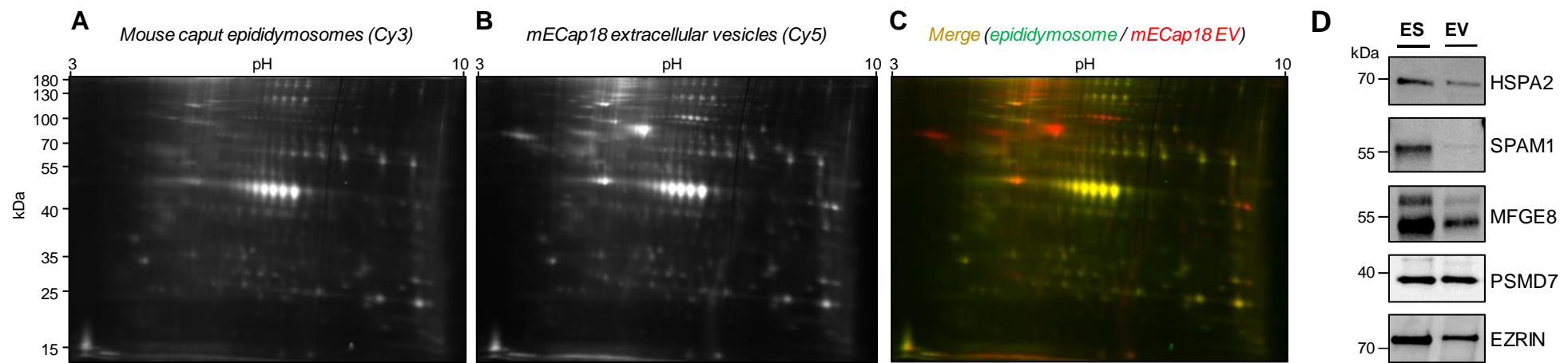


**Figure 1. Characterization of mECap18 extracellular vesicles.** mECap18 cell supernatant was fractionated by density gradient ultracentrifugation followed by the recovery and characterization of 12 equal fractions. **A)** Immunoblot analysis was performed to detect the distribution of fractions containing the characteristic EV markers of FLOT1, CD63 and CD9. **B)** On the basis of the analysis, EVs partitioning into fractions 9 - 10 were individually assessed for size heterogeneity via dynamic light scattering measurement of mean particle size. Data are reported as mean particle size (columns) and polydispersity index values (numbers above columns), whereby the lower the value the more homogenous the preparation. EVs were subsequently pooled from fractions 9 and 10, and either **C)** immobilized onto a solid support matrix consisting of aldehyde/sulphate latex beads before being sequentially labeled with anti-FLOT1 and appropriate Alexa Fluor 488 conjugated secondary antibodies (green; scale bar = 20 µm), **D)** visualized by transmission electron microscopy (scale bar = 200 nm), or **E)** prepared for immunoblotting alongside complete mECap18 cell lysates (Cell) with the EV specific markers of CD63, CD9, PSMD7, FLOT1, negative controls of HSP90B1 and APOA1 and the loading control of GAPDH. All experiments were replicated three times on independent samples and depicted are representative images and immunoblots.

Complementary ultrastructural analysis confirmed the spherical morphology, purity and size (~100 nm) of the EV preparations (Fig. 1D). Similarly, immunoblotting revealed the selective accumulation of EV markers (CD63 and CD9) relative to that of the parent mECap18 cells, the presence of additional EV markers (PSMD7, FLOT1 and GAPDH), and the absence of non-EV proteins (HSP90B1 and APOA1) (Fig. 1E) [22]. As an extension of this analysis, 2D SDS-PAGE demonstrated that the protein cargo encapsulated within mECap18 EVs is broadly similar to that of native mouse caput epididymosomes (Fig. 2A-C); a result confirmed by immunoblotting detection of prominent epididymosome proteins (Fig. 2D) [14].

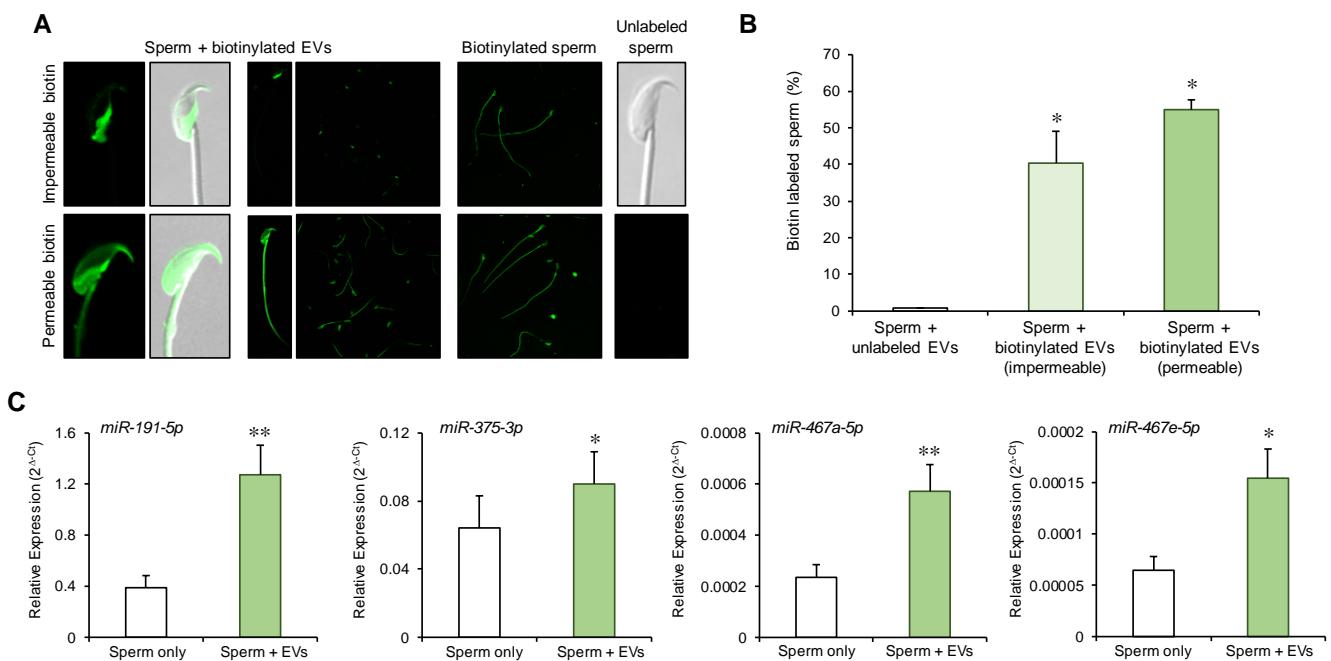
#### **mECap18 extracellular vesicles selectively interact with mouse spermatozoa *in vitro***

In keeping with conserved physical and compositional properties among mECap18 EVs and epididymosomes, we next demonstrated these vesicles also share comparable functional characteristics. Specifically, differential labeling of mECap18 EVs with membrane impermeable and permeable biotinylation reagents confirmed that they are capable of transferring encapsulated protein cargo to recipient mouse spermatozoa. Thus, EVs pre-labeled with membrane impermeable biotin relayed protein to the post-acrosomal domain of ~40% of the sperm population (Fig. 3A,B).



**Figure 2. Conservation of mECap18 extracellular vesicles and mouse epididymosome proteomes.** **A-C)** The proteomic profile of mECap18 EVs (Cy5, red) and mouse caput epididymosomes (Cy3, green) was assessed via labeling of protein extracts with size and charge matched cyanine-dyes. Labeled proteins were mixed in equal proportion, resolved by 2D SDS-PAGE and visualized. **D)** Immunoblots of mECap18 EVs and mouse caput epididymosomes were probed with antibodies against abundant epididymosome proteins. All experiments were replicated three times on independent samples and depicted are representative gel images and immunoblots.

Equivalent patterns of protein transfer were detected in spermatozoa co-incubated with EVs pre-labeled with membrane permeable biotin, however, this was accompanied by additional foci of labeling extending into the anterior portion of the sperm head and distally into the flagellum (Fig. 3A,B). The specificity of protein transfer was attested by the complete absence of biotin staining in negative control populations of naïve sperm and in those cells incubated with non-biotinylated EVs (Fig. 3A). Conversely, direct biotinylation of spermatozoa yielded uniform labeling of all cells (Fig. 3A). We also confirmed that the macromolecular exchange mediated by mECap18 EVs extended beyond their protein cargo to encompass the miRNA sub-class of sncRNA. In this context, RT-qPCR revealed a significant increase in the abundance of several target miRNAs in spermatozoa post-incubation with mECap18 extracellular vesicles (Fig. 3C). Notably, an equivalent profile of miRNAs has previously been shown to be trafficked to sperm via epididymosomes<sup>[7]</sup>, which together with conserved patterns of protein deposition, identify key functional parallels between mECap18 EVs and epididymosomes.



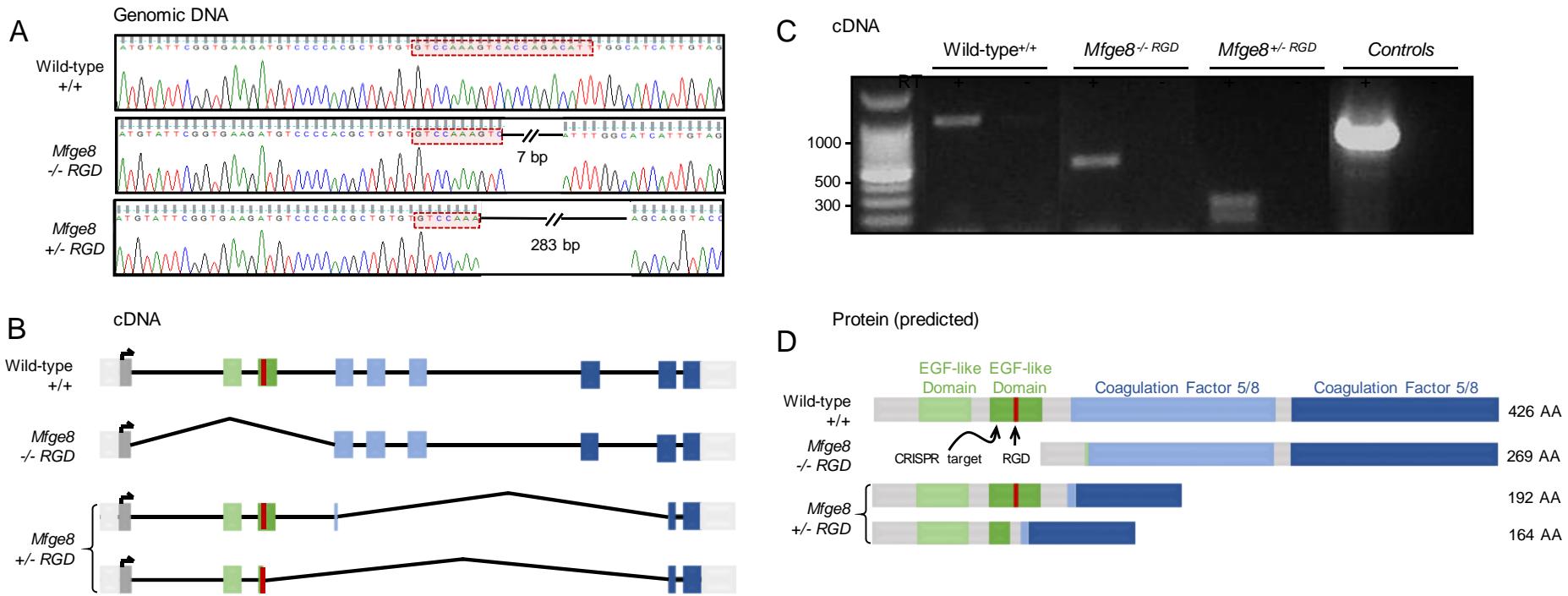
**Figure 3. mECap18 extracellular vesicle interaction with mouse spermatozoa.** **A)** The exchange of proteins from mECap18 EVs to mouse spermatozoa was assessed via pre-labeling EVs with either membrane permeable or impermeable biotinylation reagents. After co-incubation, spermatozoa were assessed for protein uptake via affinity labeling with Alexa

Fluor 488 conjugated streptavidin. Controls included spermatozoa incubated without EVs, with these cells either being left in an unlabeled state to serve as negative controls or directly labeled with biotin to confirm the specificity of EV-mediated protein transfer. **B)** Protein transfer was quantified by assessing the number of spermatozoa displaying fluorescent labeling. **C)** Transfer of alternate EV cargo, in the form of miRNAs, was also assessed via the extraction of total sperm RNA pre- and post-incubation followed by RT-qPCR amplification of target miRNAs. All graphical data are presented as means  $\pm$  SEM. Expression levels of target miRNAs were normalized against the U6 small nuclear RNA control. \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$

### Targeted mutation of the *Mfge8* gene in mECap18 cells

The demonstration that mECap18 EVs replicate the functional properties of epididymosomes prompted the use of this model to explore the cognate receptor-ligand(s) that underpin sperm-EV interaction. Target selection for this study was, in part, guided by our previous work demonstrating that antibody masking of epididymosome-resident MFGE8 effectively reduces the ability of these vesicles to bind mouse spermatozoa<sup>[14]</sup>. CRISPR-Cas9 genome-editing was therefore used to generate mutant mECap18 cells harboring deletions in key sequences in the *Mfge8* gene that encode the functional domains of the MFGE8 protein. The ligand depleted EVs secreted by two such mutant cell lines were selected for further analysis.

Sequencing of these mutants demonstrated they harbored genomic deletions of either 7 base-pairs (bp) or 283 bp compared to the wild-type *Mfge8* gene; hereafter referred to as *Mfge8*<sup>-/-RGD</sup> and *Mfge8*<sup>+/-RGD</sup>, respectively (Fig. 4A). Both genomic deletions led to significant changes in the resultant transcripts, indicating that these deletions not only removed target sequence but also altered splicing outcomes. Amplification and sequencing of the *Mfge8* cDNAs from mutant cell lines revealed that the *Mfge8*<sup>-/-RGD</sup> allele led to a complete deletion of exons 2 and 3 of the *Mfge8* gene (Fig. 4B,C and Supplementary Figure S1) and resulted in a single truncated protein product lacking both the EGF-like domains, and importantly, an integrin-binding RGD tripeptide motif (Fig. 4D). By contrast, the mutated *Mfge8*<sup>+/-RGD</sup> gene was determined to result in deletions spanning exons 3 to 7, which yielded alternatively spliced products (Fig. 4B,C and Supplementary Figure S1); the longer of which retained the RGD domain, while both products harbored truncated coagulation factor 5/8 domains (Fig. 4D).

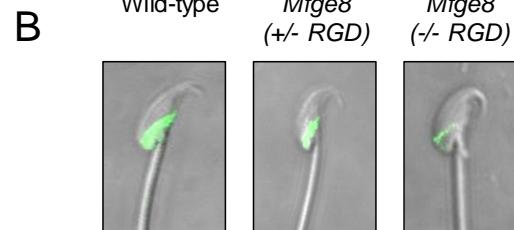
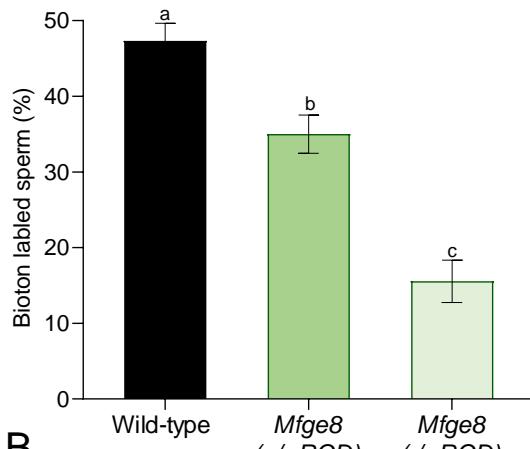


**Figure 4. Characterization of CRISPR/Cas9 mediated *Mfge8* mutations.** mECap18 cells were transfected with a 20 bp Trueguide gRNA targeting the center of exon 3 of *Mfge8*, upstream of the RGD motif. Individual clones were isolated and grown using a limited dilution strategy and subsequently sequenced to determine genotype. **A)** Resultant genomic DNA sequencing flanking the gRNA target (red box) enabled identification of two mutant lines with 7 bp and 283 bp genomic deletions (*Mfge8*<sup>-/- RGD</sup> and *Mfge8*<sup>+/+ RGD</sup>), respectively compared to wild-type (+/+). **B)** Intron/Exon mapping generated from Sanger sequencing of subcloned cDNA amplicons revealed complete deletion of exons 2 and 3 from *Mfge8*<sup>-/- RGD</sup> and a deletion spanning exons 3 to 7 from the alternately spliced *Mfge8*<sup>+/+ RGD</sup> mutant cell lines. **C)** Resolution of the mutant cDNA by agarose gel electrophoresis revealed amplicons of the expected size. **D)** Schematic of the predicted domain structure for MFGE8 protein products derived from CRISPR/Cas9 gene editing.

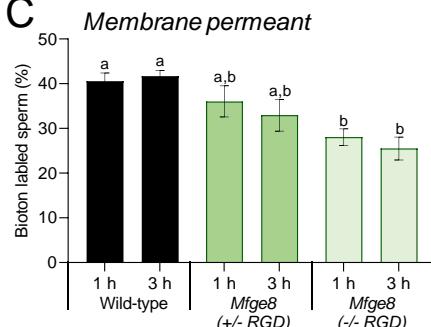
## **Loss of the MFGE8 RGD binding motif compromises sperm-extracellular vesicle interaction**

To examine whether the introduced *Mfge8* mutations had any bearing on the efficacy of sperm-EV interaction, ligand depleted EVs pre-labeled with membrane impermeable biotin were co-incubated with caput spermatozoa. Notably, both *Mfge8*<sup>-/-RGD</sup> and *Mfge8*<sup>+/+RGD</sup> mutations significantly ( $P \leq 0.05$ ) compromised the efficacy of sperm-EV interaction (Fig. 5A). This result was most pronounced in EVs totally lacking an MFGE8 RGD domain; *Mfge8*<sup>-/-RGD</sup>, which transferred biotin label to ~60% fewer spermatozoa than that of EVs recovered from wild-type mECap18 cells. Moreover, biotinylated protein transfer from *Mfge8*<sup>-/-RGD</sup> EVs was restricted to the sub-acrosomic ring (Fig. 5B), distinct from the post-acrosomal labeling witnessed in EVs generated by either wild-type or *Mfge8*<sup>+/+RGD</sup> mECap18 cells. Although not as pronounced, the transfer of *Mfge8*<sup>-/-RGD</sup> EV proteins labeled with membrane permeable biotin (i.e. comprising both encapsulated proteins and those expressed on the vesicle membrane) was also significantly reduced compared to that of wild-type EV proteins at both 1 and 3 h post-incubation (Fig 5C). Furthermore, when considering the fate of these EV proteins, it was revealed that those originating from *Mfge8*<sup>-/-RGD</sup> EVs showed minimal redistribution beyond their deposition into the post-acrosomal domain of the sperm head during the 3 h co-incubation period (Fig. 5D). By contrast, those proteins trafficked to spermatozoa via wild-type or *Mfge8*<sup>+/+RGD</sup> EVs were shown to undergo a pronounced redistribution throughout the sperm head between 1 and 3 h post-incubation [as has previously been shown for epididymosome proteins<sup>[10]</sup>].

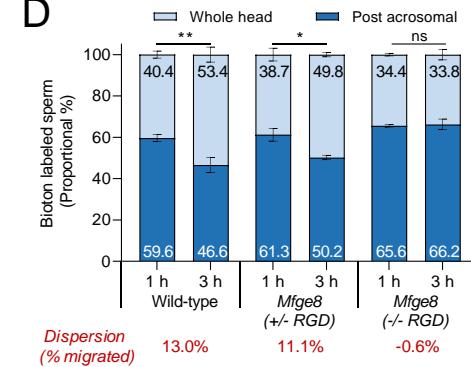
**A** *Membrane impermeant*



**C** *Membrane permeant*



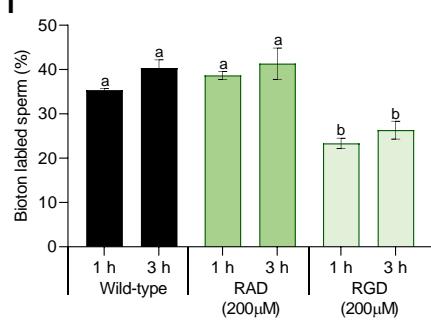
**D**



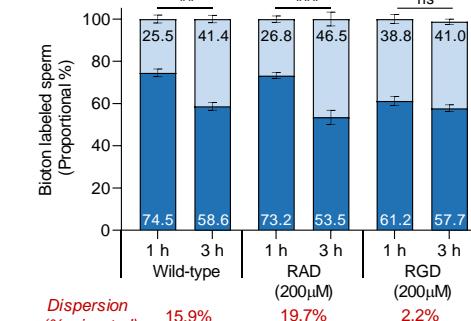
**E**



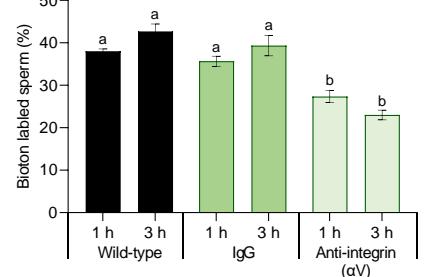
**F**



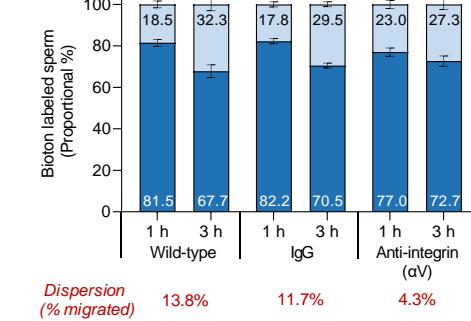
**G**



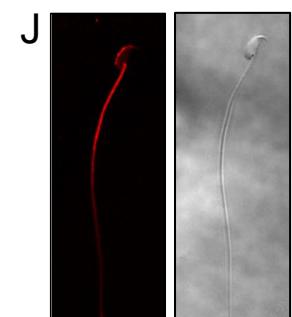
**H**



**I**



**J**



**Figure 5. Characterization of the role of MFGE8 – integrin  $\alpha$ V in sperm extracellular vesicle interactions.** EVs generated from wild-type and mutant *Mfge8* mECap18 cells, were pre-labeled with either **(A-B)** membrane impermeable or **(C-E)** membrane permeable biotin prior to co-incubation with caput epididymal spermatozoa to assess the efficacy of sperm-EV interaction. **A-E)** Spermatozoa harboring biotin label were quantified, and the relative distribution of label assessed post-incubation times of 1 and 3 h. **F-G)** To examine the role of the RGD motif in sperm-EV interaction, caput epididymal spermatozoa were pre-treated with either an RGD or RAD control tripeptide before introduction of EVs harvested from wild-type mECap18 cells. The sperm were then assessed for biotin uptake and distribution. **H-I)** Alternatively, the role of  $\alpha$ V integrin in sperm-EV adhesion was evaluated using a similar strategy in which sperm were pre-labeled with either an  $\alpha$ V function blocking antibody or non-immune IgG. **J)** Immunofluorescence was utilized to examine the presence and distribution of  $\alpha$ V integrin in mouse spermatozoa. All graphical data are presented as means  $\pm$  SEM, statistical significance was determined using either a **A)** one-way ANOVA or **C-D, F-I)** a two-way ANOVA with Tukey or Šídák correction for the appropriate multiple comparisons. **A, C, F, H)** Statistical differences of  $P \leq 0.05$  are denoted by differing letters. **D, G, I)** \*  $P \leq 0.05$  \*\*  $P \leq 0.01$  \*\*\*  $P \leq 0.001$ .

Based on these collective data we infer that, *Mfge8*<sup>-/-RGD</sup> EVs display a reduced ability to engage the downstream signaling/fusion machinery needed to permit efficient uptake of their encapsulated cargo. Moreover, the absence of an equivalent response in MFGE8<sup>+-/-RGD</sup> EVs, implicates the RGD motif as being a key mediator of this phenomenon. Accordingly, pre-absorption of sperm receptors with the RGD tripeptide proved effective in significantly attenuating the subsequent uptake (Fig. 5F), and the redistribution (Fig. 5G), of biotinylated proteins following co-incubation with wild-type EVs. By comparison, pre-incubation of spermatozoa with an equivalent amount of RAD control tripeptide had no effect on the transfer or redistribution of EV proteins (Fig. 5F,G).

### $\alpha$ V integrins participate in sperm-extracellular vesicle interaction

Whilst  $\alpha$ V integrins have been implicated as putative receptors for both the MFGE8 RGD motif<sup>[24]</sup>, and the tethering of oviductal derived EVs to spermatozoa<sup>[25]</sup>, it is not known if these receptors also mediate the equivalent adhesion of epididymal EVs to spermatozoa. Here, we demonstrate the presence and distribution of  $\alpha$ V integrin in mouse spermatozoa, revealing that the protein localizes to the peri-acrosomal domain and mid-piece of the tail in fixed permeabilized cells (Fig. 5J). Despite this localization profile differing from that of the initial site of EV adhesion, pre-labeling of mouse spermatozoa with an  $\alpha$ V function blocking antibody

(RMV-7) [26], resulted in a significant reduction in biotinylated protein transfer from wild-type EVs after 1 h (Fig. 5H). This strategy also reduced the redistribution of biotinylated EV protein after 3 h of co-incubation (Fig. 5I) compared with mock assays (i.e. sperm pre-incubated with PBS). The specificity of this response was attested by the absence of an effect in sperm pre-labeled with non-immune IgG (Fig. 5H,I).

## DISCUSSION

There is mounting evidence that epididymosomes play a fundamental role in modulating sperm function via the exchange of fertility modulating proteins and epigenetic cargo [3]. Nevertheless, the mechanisms that facilitate the selective adhesion and subsequent uptake of the epididymosome payload into recipient spermatzoa have yet to be fully resolved. There is however, general consensus that, as with other forms of EVs, the initial binding of epididymosomes is mediated by cellular protein receptors, and their cognate ligand(s) on the vesicle membrane; with our previous work having implicated MGE8 as one such ligand [14]. Accordingly, ultrastructural analyses demonstrated that MGE8 localization extends from the epididymosome surface into stalk-like projections associated with sites of epididymosome-sperm interaction [14]. Furthermore, antibody masking of MGE8 ligands compromises the efficiency of epididymosome-mediated protein transfer to recipient spermatzoa [14]. Here, we provide critical new evidence implicating MGE8 in the efficient exchange of macromolecular EV cargo to mouse spermatzoa, with our data identifying the RGD motif as being of particular importance in mediating this interaction.

MGE8 is synthesized as a ~53 kDa glycoprotein possessing a cleavable signal peptide, followed immediately by two N-terminal epidermal growth factor (EGF)-like repeats and two C-terminal discoidin/F5/8C domains. The second EGF domain also contains the RGD integrin-binding motif, which is known to engage  $\alpha\beta3/5$  integrin heterodimers to facilitate cell adhesion and induce integrin-mediated signal transduction [17]. In the mouse, MGE8 is also produced as a ~66 kDa splice variant that includes an additional 37 amino acid

proline/threonine-rich sequence, which may increase the binding efficiency to phospholipids and/or increase the efficiency of secretion. Expression of the two splice variants shows spatial and temporal specificity, with both isoforms being produced in the epididymis and represented in epididymosomes and mECap EVs. This is in contrast to the exclusive secretion of the long isoform that has previously been described for epidermal keratinocytes [27].

In terms of its function within the male reproductive tract, MFGE8 was originally characterized as a sperm protein with binding affinity for zona pellucida glycoproteins [18]. However, MFGE8 has also been implicated in the support of epididymal cell adhesion via RGD binding to αV integrin receptors on epididymal epithelial cells; a finding that accounts for the pronounced epididymal epithelium pathologies that accompany the loss of MFGE8 function [24, 28]. In spermatozoa, we hypothesize that the RGD-αV integrin interaction may stimulate intracellular signaling pathways that underpin the recruitment of lipid rafts, and their associated fusion machinery, to sites of EV docking. Indeed, we have previously shown that co-incubation of caput spermatozoa with epididymosomes can promote sequestration of lipid raft markers within the post-acrosomal domain [10]. In addition to offering insight into the receptors that putatively drive this translocation, our studies also raise the intriguing prospect that deficits in ZP adhesion documented in *Mfge8* null males [19] may, at least in part, be attributed to inefficient transfer / uptake of epididymosome cargo as opposed to resting entirely with a defect in MFGE8. Notably, the presence of MFGE8 orthologues, each possessing conserved RGD domains, in other species (such as the human, rat, bovine and porcine; Supplementary Figure S2) raises the prospect that this protein may fulfil equivalent functional roles across the mammalian lineage.

Nevertheless, whilst our data implicates MFGE8 in EV interaction, they also suggest that this protein is unlikely to be the sole ligand responsible for regulating EV adhesion. Thus, our collective interventional data point to a suppression, as opposed to elimination, of EV interaction by blocking of MFGE8 function. These data accord with independent evidence that MFGE8 represents a virtually ubiquitously membrane component of exosomes secreted from

all cell types thus far examined [29]; including mammary fat globules released into milk, as well as the epididymosomes secreted from non-rodent species [30]. It is therefore considered likely that alternative ligands are important in guiding the specificity, and perhaps efficiency, of sperm-epididymosome interactions. In this sense, it has been shown that exosomes isolated from MFGE8-deficient mice are nearly as efficient in transferring antigen/MHC complexes to recipient dendritic cells as vesicles isolated from wild-type mice [31]. Thus, despite the adhesive properties of MFGE8, the protein has been proposed to fulfil ancillary roles in terms of the formation and/or secretion of EVs. Consistent with this model, transfection of COS-7 cells with the full-length *Mfge8* coding sequence increases exosome secretion by a factor of 3- to 4-fold [32]. Conversely, expression of mutant constructs with either the C-terminal F5/8C domain deleted, abolished both the cell-surface and exosome localizations of the MFGE8 protein, whilst the C2 domain has been demonstrated essential for increased exosome secretion [32].

Notwithstanding these data, our collective evidence suggests that MFGE8 association with exosomes has additional functional significance in terms of supporting EV adhesion and/or uptake at the surface of recipient cells. Indeed, the broad conservation of MFGE8 among exosomes of varied cellular origin may account for why spermatozoa are able to interact in an efficient manner with exosomes isolated from non-reproductive cell lines such as those derived from the human embryonic kidney (i.e. HEK293T cells) [33]. In this sense, HEK293T exosomes have been shown to be internalized into boar spermatozoa within as little as 10 min of co-incubation; with no significant deleterious effects on basic sperm function [33]. Such findings raise the intriguing prospect of being able to harness EVs, laden with cell adhesion molecules such as MFGE8, as versatile research tools for studying sperm biology and as a prescient for developing vectors capable of delivering therapeutic payloads for the treatment of male infertility.

## **DECLARATIONS OF INTEREST**

The authors have no competing interests to declare

## **AUTHOR CONTRIBUTIONS**

NAT, SJS, WZ, DAS-B, PS performed the experimental work and data analysis. NAT, MDD, ALE, GND, EGB, SDR, and BN each contributed to the conceptualization, literature curation, and drafting of the manuscript. All authors edited the manuscript and approved the final version of the manuscript.

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## A) Mutant *Mfge8* genomic DNA sequence alignment

WT +/+	CGGTTTCACAGTGGATGCCGAGTAGCCCACAGGGCACTGGCAGATGTATTCCGTGAAGA	Exon 3
-/- RGD	CGGTTTCACAGTGGATGCCGAGTAGCCCACAGGGCACTGGCAGATGTATTCCGTGAAGA	
+/- RGD	CGGTTTCACAGTGGATGCCGAGTAGCCCACAGGGCACTGGCAGATGTATTCCGTGAAGA	
	*****	
WT +/+	TGTCCCCACGCTGTGTGTCAAAGTC-----ATTGGCATCATGTAGCAAGGGTTG	sgRNA target
-/- RGD	TGTCCCCACGCTGTGTGTCAAAGTC-----ATTGGCATCATGTAGCAAGGGTTG	
+/- RGD	TGTCCCCACGCTGTGTGTCAA-----	
	*****	
WT +/+	GGGAGCATGGCCTGAAAGCGAGGAGACACATCAGATGAAGAGGTGGCTAAAAGGGCT	
-/- RGD	GGGAGCATGGCCTGAAAGCGAGGAGACACATCAGATGGAGAGGTGGCTAAAAGGGCT	
+/- RGD	-----	
WT +/+	GGGAACACAGCAAAGGGCAGCACGTGACCCAGCCTCCAGCGATCCATCCAATGAGTC	
-/- RGD	GGGAACACAGCAAAGGGCAGCACGTGACCCAGCCTCCAGCGATCCATCCAATGAGTC	
+/- RGD	-----	
WT +/+	TTTTCTCCCCGCCACCACCATCCTGGACCCAAGAGGGTCTCTGAAAGGATCAGTTGCC	
-/- RGD	TTTTCTCCCCGCCACCACCATCCTGGACCCAAGAGGGTCTCTGAAAGGATCAGTTGCC	
+/- RGD	-----	
WT +/+	CACATACTGAAATCATTGAAACAAGGGTACCCACTTGGAAAGAGACAGGGGAGGCAGGCC	
-/- RGD	CACATACTGAAATCATTGAAACAAGGGTACCCACTTGGAAAGAGACAGGGGAGGCAGGCC	
+/- RGD	-----	
WT +/+	ATGGGGAGCAGGTACCTCTCAGTCTCATTGCACACAAGGCCTGTGAAGCCTTCAGGGC	Exon 2
-/- RGD	ATGGGGAGCAGGTACCTCTCAGTCTCATTGCACACAAGGCCTGTGAAGCCTTCAGGGC	
+/- RGD	-----AGCAGGTACCTCTCAGTCTCATTGCACACAAGGCCTGTGAAGCCTTCAGGGC	
	*****	
WT +/+	AGAGGCAGTAGATGTCATTGCTTGGCCCGTCAAGCAGGTGCCACCGTTCAGGCACAGGC	
-/- RGD	AGAGGCAGTAGATGTCATTGCTTGGCCCGTCAAGCAGGTGCCACCGTTCAGGCACAGGC	
+/- RGD	AGAGGCAGTAGATGTCATTGCTTGGCCCGTCAAGCAGGTGCCACCGTTCAGGCACAGGC	
	*****	
WT +/+	TGGAGTCACAGAACGTACCTGCAACCACAGACCAAACATGATCATTGTAAGCACTAA	
-/- RGD	TGGAGTCACAGAACGTACCTGCAACCACAGACCAAACATGATCATTGTAAGCACTAA	
+/- RGD	TGGAGTCACAGAACGTACCTGCAACCACAGACCAAACATGATCATTGTAAGCACTAA	
	*****	
WT +/+	GAGTCCCCTACCAA	
-/- RGD	GAGTCCCCTACCAA	
+/- RGD	GAGTCCCCTACCAA	
	*****	

## B) Mutant *Mfge8* cDNA sequence alignment

WT +/+	ATGCAGGTCTCCCGTGTGCTGGCCCGCCTGTGCGGCATGCTACTCTGCGCCTCTGGCCTC	Exon 1
-/- RGD	ATGCAGGTCTCCCGTGTGCTGGCCCGCCTGTGCGGCATGCTACTCTGCGCCTCTGGCCTC	
+/- RGD	ATGCAGGTCTCCCGTGTGCTGGCCCGCCTGTGCGGCATGCTACTCTGCGCCTCTGGCCTC	
+/- RGD	ATGCAGGTCTCCCGTGTGCTGGCCCGCCTGTGCGGCATGCTACTCTGCGCCTCTGGCCTC	
	*****	
WT +/+	TTCGCCCGGTCTGGTACTTCTGTGACTCCAGCCTGTGCCTGAACGGTGGCACCTGCTTG	Exon 2
-/- RGD	TTCGCCCGGTCTG-----	
+/- RGD	TTCGCCCGGTCTGGTACTTCTGTGACTCCAGCCTGTGCCTGAACGGTGGCACCTGCTTG	
+/- RGD	TTCGCCCGGTCTGGTACTTCTGTGACTCCAGCCTGTGCCTGAACGGTGGCACCTGCTTG	
	120	
	72	
	120	
	120	
WT +/+	ACGGGCCAAGACAATGACATCTACTGCCCCTGCCCCTGAAGGCTTCACAGGCCCTGTGTC	180

-/- RGD	-----	72
+/- RGD	ACGGGCCAAGACAATGACATCTACTGCCCTGCCCTGAAGGCTTCACAGGCCCTGTGTGC	180
+/- RGD	ACGGGCCAAGACAATGACATCTACTGCCCTGCCCTGAAGGCTTCACAGGCCCTGTGTGC	180
WT +/+	AATGAGACTGAGAGAGGACCATGCTCCCCAACCCCTTGCTACAATGATGCCA <b>AATGTCTG</b>	240
-/- RGD	-----	72
+/- RGD	AATGAGACTGAGAGAGGACCATGCTCCCCAACCCCTTGCTACAATGATGCCA <b>AATGTCTG</b>	240
+/- RGD	AATGAGACTGAGAGAGGACCATGCTCCCCAACCCCTTGCTACAATGATGCCA <b>AATGTCTG</b>	240
WT +/+	<b>GTGACTTTGGAC</b> ACACAG <b>CGTGGGGAC</b> ATCTTCACCGAATACATCTGCCAGTGCCCTGTG	300
-/- RGD	-----	72
+/- RGD	<b>GTGACTTTGGAC</b> GCACAG <b>CGTGGGGAC</b> ATCTTCACCGAATACATCTGCCAGTGCCCTGTG	300
+/- RGD	<b>GTGACTTTGG</b> -----	250
WT +/+	<b>GGCTACTCGGGCATCCACTGTGAAACCGGTTGTTCTACACAGCTGGCATGGAAAGGGGC</b>	360
-/- RGD	----- <b>GTTGTTCTACACAGCTGGCATGGAAAGGGGC</b>	104
+/- RGD	<b>GGCTACTCGGGCATCCACTGTGAAACCGGTTGTTCTACACAGCTGGCATGGAAAGGGGC</b>	334
+/- RGD	-----	250
WT +/+	GCCATTGCTGATTCACAGATTCCGCCTCGTCTGTGTATATGGGTTCATGGGTTGCAG	420
-/- RGD	GCCATTGCTGATTCACAGATTCCGCCTCGTCTGTGTATATGGGTTCATGGGTTGCAG	164
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	CGCTGGGGCCCGGAGCTGGCTCGTCTGTACCGCACAGGGATCGTCAATGCCCTGGACAGCC	480
-/- RGD	CGCTGGGGCCCGGAGCTGGCTCGTCTGTACCGCACAGGGATCGTCAATGCCCTGGACAGCC	224
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	AGCAACTATGATAGCAAGCCCTGGATCCAGGTGAACCTTCTGCCGAAGATGCCGTATCA	540
-/- RGD	AGCAACTATGATAGCAAGCCCTGGATCCAGGTGAACCTTCTGCCGAAGATGCCGTATCA	284
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	<b>GGTGTGATGACGCAGGGTGCCAGCCGTGCCGGAGGGCGGAGTACCTGAAGACCTTCAAG</b>	600
-/- RGD	<b>GGTGTGATGACGCAGGGTGCCAGCCGTGCCGGAGGGCGGAGTACCTGAAGACCTTCAAG</b>	344
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	<b>GTGGCTTACAGCCTCGACGGACGCAAGTTGAGTTCATCCAGGATGAAAGCGGTGGAGAC</b>	660
-/- RGD	<b>GTGGCTTACAGCCTCGACGGACGCAAGTTGAGTTCATCCAGGATGAAAGCGGTGGAGAC</b>	404
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	<b>AAGGAGTTTGGTAACCTGGACAACAAACAGCCTGAAGGTTAACATGTTCAACCCGACT</b>	720
-/- RGD	<b>AAGGAGTTTGGTAACCTGGACAACAAACAGCCTGAAGGTTAACATGTTCAACCCGACT</b>	464
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	CTGGAGGCACAGTACATAAAGCTGTACCTGTTCTGTGCCACCGCGGCTGCACCCCTCCGC	780
-/- RGD	CTGGAGGCACAGTACATAAAGCTGTACCTGTTCTGTGCCACCGCGGCTGCACCCCTCCGC	524
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	TTCGAGCTCTGGGCTGTGAGTTGCACGGATGTTCTGAGCCCTGGGCTGAAGAATAAC	840
-/- RGD	TTCGAGCTCTGGGCTGTGAGTTGCACGGATGTTCTGAGCCCTGGGCTGAAGAATAAC	584
+/- RGD	-----	334
+/- RGD	-----	250
WT +/+	ACAATT CCTGACAGCCAGATGTCAGCCTCCAGCAGCTACAAGACATGGAACCTGCGTGCT	900
-/- RGD	ACAATT CCTGACAGCCAGATGTCAGCCTCCAGCAGCTACAAGACATGGAACCTGCGTGCT	644
+/- RGD	-----	334
+/- RGD	-----	250

Exon 3

sgRNA

RGD

Exon 4

Exon 5

Exon 6

Exon 7

WT	+/+	TTTGGCTGGTACCCCCACTGGGAAGGCTGGATAATCAGGGCAAGATCAATGCCCTGGACG	960
-/-	RGD	TTTGGCTGGTACCCCCACTGGGAAGGCTGGATAATCAGGGCAAGATCAATGCCCTGGACG	704
+/-	RGD	-----	334
+/-	RGD	-----	250
WT	+/+	GCTCAGAGCAACAGTGCAAGGAATGGCTGCAGGTTGACCTGGGCACTCAGAGGCAAGTG	1020
-/-	RGD	GCTCAGAGCAACAGTGCAAGGAATGGCTGCAGGTTGACCTGGGCACTCAGAGGCAAGTG	764
+/-	RGD	-----	334
+/-	RGD	-----	250
WT	+/+	ACAGGAATCATCACCCAGGGGCCGTGACTTGGCCACATCCAGTATGTGGCGTCCTAC	1080
-/-	RGD	ACAGGAATCATCACCCAGGGGCCGTGACTTGGCCACATCCAGTATGTGGCGTCCTAC	824
+/-	RGD	-----	359
+/-	RGD	-----	275
WT	+/+	AAGGTAGCCCACAGTGATGATGGTGTGCAGTGGACTGTATATGAGGAGCAAGGAAGCAGC	1140
-/-	RGD	AAGGTAGCCCACAGTGATGATGGTGTGCAGTGGACTGTATATGAGGAGCAAGGAAGCAGC	884
+/-	RGD	AAGGTAGCCCACAGTGATGATGGTGTGCAGTGGACTGTATATGAGGAGCAAGGAAGCAGC	419
+/-	RGD	AAGGTAGCCCACAGTGATGATGGTGTGCAGTGGACTGTATATGAGGAGCAAGGAAGCAGC	335
WT	+/+	AAGGTCTCCAGGGCAACTTGGACAACAACCTCCACAAGAAGAACATCTCGAGAAACCC	1200
-/-	RGD	AAGGTCTCCAGGGCAACTTGGACAACAACCTCCACAAGAAGAACATCTCGAGAAACCC	944
+/-	RGD	AAGGTCTCCAGGGCAACTTGGACAACAACCTCCACAAGAAGAACATCTCGAGAAACCC	479
+/-	RGD	AAGGTCTCCAGGGCAACTTGGACAACAACCTCCACAAGAAGAACATCTCGAGAAACCC	395
WT	+/+	TTCATGGCTCGCTACGTGCGTGTCTTCAGTGTCTGGCATACCGCATCACCTGCGC	1260
-/-	RGD	TTCATGGCTCGCTACGTGCGTGTCTTCAGTGTCTGGCATACCGCATCACCTGCGC	1004
+/-	RGD	TTCATGGCTCGCTACGTGCGTGTCTTCAGTGTCTGGCATACCGCATCACCTGCGC	539
+/-	RGD	TTCATGGCTCGCTACGTGCGTGTCTTCAGTGTCTGGCATACCGCATCACCTGCGC	455
WT	+/+	CTGGAGCTGCTGGGCTGTTAATGCTCAGTCCTGCCAGGCCAACAGATGAGGATGGCCAGA	1320
-/-	RGD	CTGGAGCTGCTGGGCTGTTAATGCTCAGTCCTGCCAGGCCAACAGATGAGGATGGCCAGA	1064
+/-	RGD	CTGGAGCTGCTGGGCTGTTAATGCTCAGTCCTGCCAGGCCAACAGATGAGGATGGCCAGA	599
+/-	RGD	CTGGAGCTGCTGGGCTGTTAATGCTCAGTCCTGCCAGGCCAACAGATGAGGATGGCCAGA	515
WT	+/+	GGCTGAGGGGCCTCTGCCCTGCCCTCCAGGCCCTGCTGCCCTCTGTGGCTGACGACCT	1380
-/-	RGD	GGCTGAGGGGCCTCTGCCCTGCCCTCCAGGCCCTGCTGCCCTCTGTGGCTGACGACCT	1124
+/-	RGD	GGCTGAGGGGCCTCTGCCCTGCCCTCCAGGCCCTGCTGCCCTCTGTGGCTGACGACCT	659
+/-	RGD	GGCTGAGGGGCCTCTGCCCTGCCCTCCAGGCCCTGCTGCCCTCTGTGGCTGACGACCT	575
WT	+/+	TCTTGGCCTTCCCTTGATTGTACTGGGCTGGAGGCAGGAAGGCCAGGGGATTCAG	1440
-/-	RGD	TCTTGGCCTTCCCTTGATTGTACTGGGCTGGAGGCAGGAAGGCCAGGGGATTCAG	1184
+/-	RGD	TCTTGGCCTTCCCTTGATTGTACTGGGCTGGAGGCAGGAAGGCCAGGGGATTCAG	719
+/-	RGD	TCTTGGCCTTCCCTTGATTGTACTGGGCTGGAGGCAGGAAGGCCAGGGGATTCAG	635
WT	+/+	AGTTGCCCTCACCCCTTCCCTCACCTGCGACCCCCACAGGCCCTCTGTAGCCCCCTT	1500
-/-	RGD	AGTTGCCCTCACCCCTTCCCTCACCTGCGACCCCCACAGGCCCTCTGTAGCCCCCTT	1244
+/-	RGD	AGTTGCCCTCACCCCTTCCCTCACCTGCGACCCCCACAGGCCCTCTGTAGCCCCCTT	779
+/-	RGD	AGTTGCCCTCACCCCTTCCCTCACCTGCGACCCCCACAGGCCCTCTGTAGCCCCCTT	695
WT	+/+	CTCTCAGGCATTCTGGGGAGTTGGACAGGTCTGAGATGAATAGAGAAGAGTGAAGT	1560
-/-	RGD	CTCTCAGGCATTCTGGGGAGTTGGACAGGTCTGAGATGAATAGAGAAGAGTGAAGT	1304
+/-	RGD	CTCTCAGGCATTCTGGGGAGTTGGACAGGTCTGAGATGAATAGAGAAGAGTGAAGT	839
+/-	RGD	CTCTCAGGCATTCTGGGGAGTTGGACAGGTCTGAGATGAATAGAGAAGAGTGAAGT	755
WT	+/+	TGGGGTATGTGGGCTATCTGTACCAACCACCCAAGTCCTAAACTTCTGCCAGGGCTTG	1620
-/-	RGD	TGGGGTATGTGGGCTATCTGTACCAACCACCCAAGTCCTAAACTTCTGCCAGGGCTTG	1364
+/-	RGD	TGGGGTATGTGGGCTATCTGTACCAACCACCCAAGTCCTAAACTTCTGCCAGGGCTTG	899
+/-	RGD	TGGGGTATGTGGGCTATCTGTACCAACCACCCAAGTCCTAAACTTCTGCCAGGGCTTG	815
WT	+/+	ACTCAGGACTGAAGGGAGCCCCCTGACTGCCATCCCTCTGCACACCACACATTCTCC	1680
-/-	RGD	ACTCAGGACTGAAGGGAGCCCCCTGACTGCCATCCCTCTGCACACCACACATTCTCC	1424
+/-	RGD	ACTCAGGACTGAAGGGAGCCCCCTGACTGCCATCCCTCTGCACACCACACATTCTCC	959
+/-	RGD	ACTCAGGACTGAAGGGAGCCCCCTGACTGCCATCCCTCTGCACACCACACATTCTCC	875

### Exon 8

### Exon 9

*****			
WT +/+	ATGTTCCATTCCGGGAAGGGAGAGGCCACGTCCGCTTGACTGTCCTTGGGTCAACCAGGTC	1740	
-/- RGD	ATGTTCCATTCCGGGAAGGGAGAGGCCACGTCCGCTTGACTGTCCTTGGGTCAACCAGGTC	1484	
+/- RGD	ATGTTCCATTCCGGGAAGGGAGAGGCCACGTCCGCTTGACTGTCCTTGGGTCAACCAGGTC	1019	
+/- RGD	ATGTTCCATTCCGGGAAGGGAGAGGCCACGTCCGCTTGACTGTCCTTGGGTCAACCAGGTC	935	
*****			
WT +/+	CTGCCTCTTATCTCCTGAGACGCCCTTGACCCCTTGACTGGAGCCTCAGTTGACAAGGA	1800	
-/- RGD	CTGCCTCTTATCTCCTGAGACGCCCTTGACCCCTTGACTGGAGCCTCAGTTGACAAGGA	1544	
+/- RGD	CTGCCTCTTATCTCCTGAGACGCCCTTGACCCCTTGACTGGAGCCTCAGTTGACAAGGA	1079	
+/- RGD	CTGCCTCTTATCTCCTGAGACGCCCTTGACCCCTTGACTGGAGCCTCAGTTGACAAGGA	995	
*****			
WT +/+	GACTGGCGGGTCTGGAGAGGTCTGGCTCTGGGTGGTTGACAGGTTGGCTGTGGGACCT	1860	
-/- RGD	GACTGGCGGGTCTGGAGAGGTCTGGCTCTGGGTGGTTGACAGGTTGGCTGTGGGACCT	1604	
+/- RGD	GACTGGCGGGTCTGGAGAGGTCTGGCTCTGGGTGGTTGACAGGTTGGCTGTGGGACCT	1139	
+/- RGD	GACTGGCGGGTCTGGAGAGGTCTGGCTCTGGGTGGTTGACAGGTTGGCTGTGGGACCT	1055	
*****			
WT +/+	CTGCTGGCTTGCTACCAAGTTAACAGCAGATTCCAAAATACATTCTGTTCTCCACTG	1920	
-/- RGD	CTGCTGGCTTGCTACCAAGTTAACAGCAGATTCCAAAATACATTCTGTTCTCCACTG	1664	
+/- RGD	CTGCTGGCTTGCTACCAAGTTAACAGCAGATTCCAAAATACATTCTGTTCTCCACTG	1199	
+/- RGD	CTGCTGGCTTGCTACCAAGTTAACAGCAGATTCCAAAATACATTCTGTTCTCCACTG	1115	
*****			
WT +/+	GAAAAAAAAAAAAAA	1937	
-/- RGD	GAAAAAAAAAAAAAA	1681	
+/- RGD	GAAAAAAAAAAAAAA	1216	
+/- RGD	GAAAAAAAAAAAAAA	1132	
*****			

### C) Predicted MGE8 protein sequence alignment

WT +/+	MQVSRLAALCGMLLCASGLFAASGDFCDSSLCLNGGTLTGQDNDIYCLCP EGFTGLVC	60
-/- RGD	MQVSRLAALCGMLLCASGLFAASGDFCDSS-----	30
+/- RGD	MQVSRLAALCGMLLCASGLFAASGDFCDSSLCLNGGTLTGQDNDIYCLCP EGFTGLVC	60
+/- RGD	MQVSRLAALCGMLLCASGLFAASGDFCDSSLCLNGGTLTGQDNDIYCLCP EGFTGLVC	60
*****		
WT +/+	NETERGPCSPNPCYNDAKCLVLDTQRGDIFTEYICQCPVGYSIGHCETGCSTQLGMEGG	120
-/- RGD	-----	30
+/- RGD	NETERGPCSPNPCYNDAKCLVLDQAQRGDIFTEYICQCPAGYSGIGHCETGCSTS SMRPT	120
+/- RGD	NETERGPCSPNPCYNDAKCLVTLA-----TSSMRPT	92
*****		
WT +/+	AIADSQISASSVYMGFMGLQRWGP ELARLYRTGIVNAWTASNYDSKPWIQVNLLRKM RVS	180
-/- RGD	-----	30
+/- RGD	R-----	121
+/- RGD	R-----	93
*****		
WT +/+	GVMTQGASRAGRAEYLKTFKVAYS LGDRKFEFIQDESGGDKEFLGNLDNNSLKVNMFNPT	240
-/- RGD	-----SRAGRAEYLKTFKVAYS LGDRKFEFIQDESGGDKEFLGNLDNNSLKVNMFNPT	83
+/- RGD	-----	121
+/- RGD	-----	93
*****		
WT +/+	LEAQYIKLYPV SCHR GCTLRFELLGCELHGCSEPLGLKNNTIPDSQMSASSSYKTWNLRA	300
-/- RGD	LEAQYIKLYPV SCHR GCTLRFELLGCELHGCSEPLGLKNNTIPDSQMSASSSYKTWNLRA	143
+/- RGD	-----	121
+/- RGD	-----	93
*****		
WT +/+	FGWYPHLGR LDNQG KINAWTAQNSAKEWLQVDLGTQRQVTGII TQGARD FGH IQY VASY	360
-/- RGD	FGWYPHLGR LDNQG KINAWTAQNSAKEWLQVDLGTQRQVTGII TQGARD FGH IQY VASY	203
+/- RGD	-----YVASY	126
+/- RGD	-----YVASY	98

\*\*\*\*\*

WT +/+	KVAHSDDGQWTVYEEQGSSKVFQGNLDNNSHKKNIFEKPFMARYVRVLPVSWHNRLTR	420
-/- RGD	KVAHSDDGQWTVYEEQGSSKVFQGNLDNNSHKKNIFEKPFMARYVRVLPVSWHNRLTR	263
+/- RGD	KVAHSDDGQWTVYEEQGSSKVFQGNLDNNSHKKNIFEKPFMARYVRVLPVSWHNRLTR	186
+/- RGD	KVAHSDDGQWTVYEEQGSSKVFQGNLDNNSHKKNIFEKPFMARYVRVLPVSWHNRLTR	158
	*****	
WT +/+	LELLGC	426
-/- RGD	LELLGC	269
+/- RGD	LELLGC	192
+/- RGD	LELLGC	164
	*****	

**Supplementary Figure S1. Sequence analysis of *Mfge8* mutant cell lines.** mECap18 cells were transfected with a 20 bp Trueguide gRNA targeting the center of exon 3 of *Mfge8*, upstream of the RGD motif. Individual clones were isolated and grown using a limited dilution strategy and subsequently sequenced to determine genotype. **A)** Resultant genomic DNA sequencing flanking the sgRNA target of two mutant lines with 7 bp and 283 bp genomic deletions (*Mfge*<sup>-/- RGD</sup> and *Mfge8*<sup>+/- RGD</sup>), respectively compared to wild-type (+/+). Note that the reverse strand gDNA sequence is depicted. **B)** Sequencing of subcloned cDNA amplicons revealed complete deletion of exons 2 and 3 from *Mfge8*<sup>-/- RGD</sup> and a deletion spanning exons 3 to 7 from the alternately spliced *Mfge8*<sup>+/- RGD</sup> mutant cell lines. **C)** Predicted amino acid sequence of the mutant MFGE8 protein products produced by *Mfge*<sup>-/- RGD</sup> and *Mfge8*<sup>+/- RGD</sup> mECap18 cell lines.

## Supplemental Figure S2: Alignment of MGFE8 orthologues

## **Chapter 4: Final Discussion and Future Research Directions**

### **CHAPTER OVERVIEW:**

The findings described in this thesis highlight the vulnerability of the sperm sncRNA profile to environmental insult during epididymal transit. Importantly, these advances have provided insight into a novel mechanism of how paternal toxicant exposure alters the sncRNA profile of the transcriptionally silent epididymal spermatozoa, the role of epididymal EVs (epididymosomes) in shaping this sncRNA profile, and the subsequent impact on the embryo following the delivery of an altered sncRNA profile. This final chapter provides an overview of the findings presented in this thesis in the context of the current literature, identifies limitations to the described studies and presents avenues for future research. Specifically, we discuss where the field stands on establishing a mechanism of sncRNA alteration in the maturing sperm cell following paternal exposure to stress, the emerging role of sperm-borne sncRNA following fertilisation and the need to study the mechanism of EV-mediated communication owing to its evolving contribution in shaping the sperm sncRNA profile.

## **Introduction**

As the wave of human infertility continues to rise, so too has the adoption of assisted reproductive technologies (ART) in a bid to overcome this pathology. Indeed, the number of children born globally as a result of ART procedures is rapidly approaching 0.1% of the global population, with predictions estimating the number of people whose existence will have depended on ART will represent 3% of humanity by 2100 (de Mouzon *et al.*, 2020; Faddy *et al.*, 2018). The aim of ART procedures such as *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) is to remove natural selection barriers to facilitate fertilisation that would otherwise be incongruous *in vivo*. Of couples experiencing infertility and presenting at ART clinics, a male contributing factor is diagnosed in 40-50% of cases (Kumar and Singh, 2015). In a portion of these cases, semen assessment returns results within normal range despite an inability to achieve paternity naturally, thus identifying an unmet need for additional forms of assessment with which to diagnose male fertility. Furthermore, while ART procedures may result in a zygote fit for embryo transfer, from a paternal perspective, the outcome of a healthy child is not solely dependent on the ability of the spermatozoon to successfully fertilise an oocyte. Indeed, the role of spermatozoa in the reproductive process has diversified in recent years, as it is now known that sperm deliver a range of factors to the oocyte in addition to the paternal genome, which contribute to normal embryo and foetal development and offspring health outcomes. Perhaps not surprisingly, such factors are overlooked by traditional descriptive clinical assessments of the male ejaculate. While these newly identified sperm-borne factors are highly complex and likely synergistically linked, one such paternal contributor which has recently begun to receive considerable focus is that of the sncRNAs. Indeed, it is now known that the sncRNA profile of spermatozoa is dynamic and modified in response to a range of paternal environmental and lifestyle factors. Moreover, the delivery of an altered sperm sncRNA profile to the oocyte can lead to a spectrum of consequences, from delayed embryo development to offspring phenotypes, such as metabolic disturbances (Chen *et al.*, 2016; Sharma *et al.*, 2016).

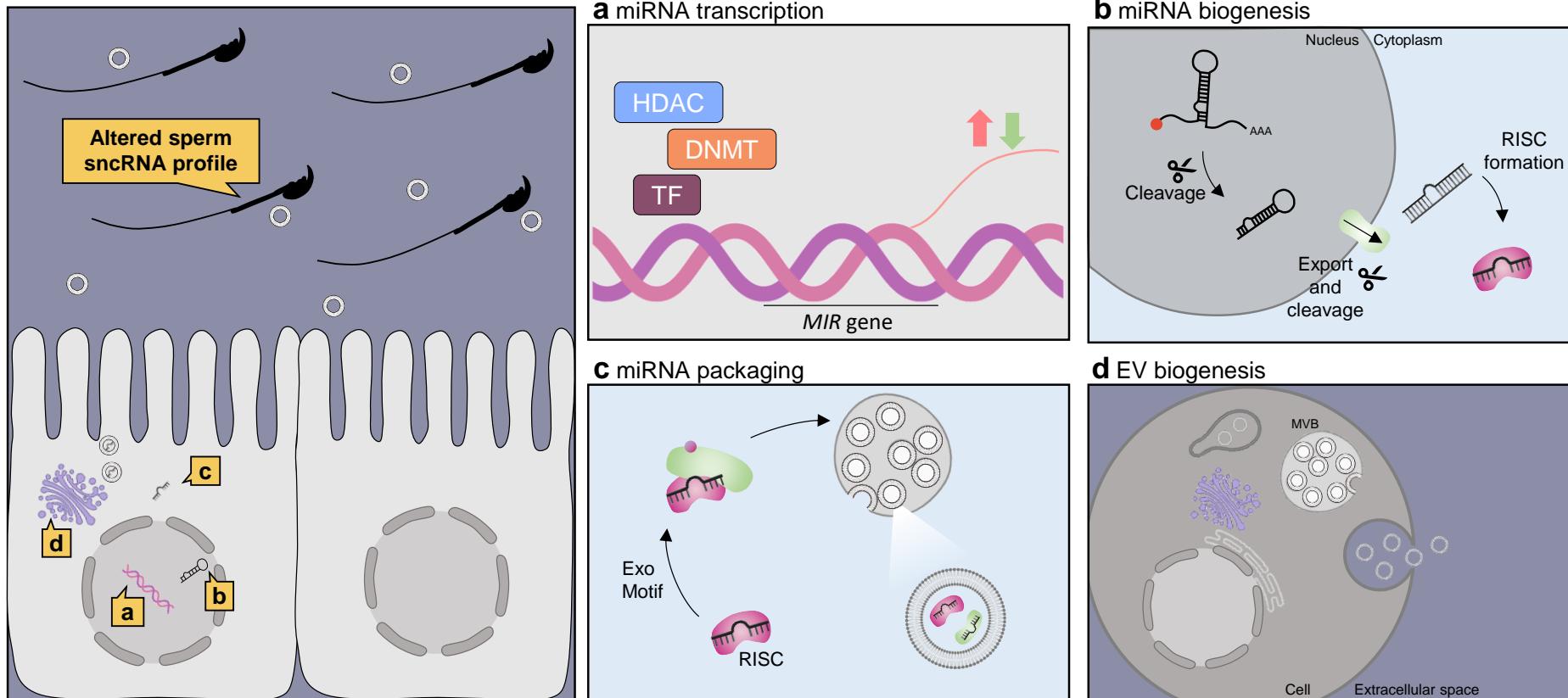
Notably, one of the leading contributors to the rise in male infertility is exposure to environmental insults, including nutritional perturbations, smoking, alcohol, toxicant exposure and stress (reviewed in (Oliva *et al.*, 2001)). Interestingly, this spectrum of exposures has also been shown to alter the composition of the sperm sncRNA profile (Chen *et al.*, 2016; Li *et al.*, 2012; Marczylo *et al.*, 2012; Rodgers *et al.*, 2013; Rompala *et al.*, 2018). Hence, the sperm sncRNA profile presents an attractive readout of paternal preconception health and may hold diagnostic value that could lift the current clinical andrology assessment to a molecular future, with the potential to improve ART success rates and healthier outcomes for the offspring. Indeed, spermatozoa from fertile individuals contain a population of miRNAs that is distinct from their infertile counterparts (Abu-Halima *et al.*, 2013; Hua *et al.*, 2019; Salas-Huetos *et al.*, 2016); a phenomenon that is also apparent in other species such as the bull (Alves *et al.*, 2019; Fagerlind *et al.*, 2015). Dissection of the dysregulated miRNAs present in the spermatozoa of infertile patients has identified specific miRNA transcripts, such as *hsa-miR-191-5p* which are associated with improved early embryo development (Xu *et al.*, 2020). While such investigations are still in their infancy, they provide promising results highlighting the value of understanding the range of paternal exposures that influence the sperm sncRNA profile to advise paternal preconception health. They also emphasise the importance of defining how paternal exposure leads to a compositional change in the sperm sncRNA profile in order to inform the design of rational therapeutic interventions.

### **Understanding the mechanistic link between paternal environmental and lifestyle experience and alteration to the sperm sncRNA profile**

Research over the past decade has catalogued a growing number of paternal environmental and lifestyle factors, ranging from a single traumatic experience early in life to chronic ethanol exposure, that are capable of influencing the sperm sncRNA profile (Trigg *et al.*, 2019) (Chapter 1). Importantly, this phenomenon has been substantiated in a number of different species including humans (Donkin *et al.*, 2016; Gapp *et al.*, 2014; Nätt *et al.*, 2019). While the list of paternal experiences that influence the sperm sncRNA profile continues to expand, what

remains less certain is the response(s) elicited within the male reproductive tract that direct changes to the sperm epigenome. To date, research has focused on the tRF subclass of sncRNA leading to the identification of key tRNA methyltransferase enzymes (Nsun2 and Dnmt2) that not only direct tRNA methylation but also influence downstream tRNA cleavage; the modulation of which has been predicted to account for altered tRF abundance in sperm (Rompala *et al.*, 2018; Zhang *et al.*, 2018). This targeted approach however, does not rule out alternate pathways that may be dysregulated and exert additional influence over sperm tRF abundance.

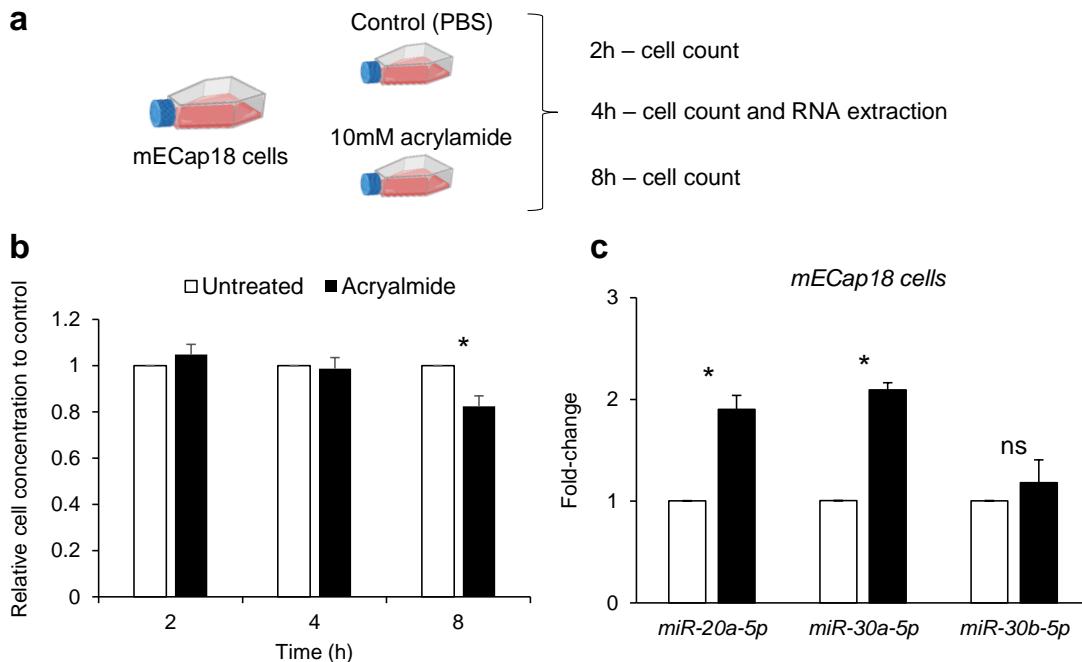
To explore this possibility, we have examined the global proteomic response of epididymal epithelial cells following exposure to acrylamide with the goal of identifying mechanism(s) that may underpin changes to the sperm sncRNA profile (Chapter 2). To our knowledge, this proteomic analysis has provided the greatest depth of protein identification for isolated epididymal epithelial cells to date, with a core proteome consisting of 4,405 proteins. Moreover, the application of quantitative mass spectrometry has enabled identification of pathways dysregulated by acrylamide exposure that could conceivably lead to a change in the sncRNA profile of the sperm harboured within the epididymal lumen. Focusing on the miRNA subclass of sncRNA, we readily concede that the sperm miRNA profile could be altered through a number of different pathways, including i) transcriptional regulation of miRNA, ii) miRNA production and processing iii) packaging of miRNA into EVs and/or iv) the biogenesis, release and uptake of EVs by recipient spermatozoa (Fig 1). Studies to date have identified the regulation of miRNA expression in the epididymis following conditions of stress occurs primarily at the level of transcriptional control (Fig 1a) (Chan *et al.*, 2020). This contrasts the control of tRF abundance in mature spermatozoa, which instead appears to be regulated at the level of their biogenesis (Fig 1b) (Rompala *et al.*, 2018; Zhang *et al.*, 2018). Whether or not this disparity in the level of regulation targeted for different subclasses of sncRNA will be upheld following further investigation remains a matter of speculation.



**Figure 1: Potential mechanisms of sperm miRNA composition change following paternal exposure to environmental or lifestyle factors.** Dysregulation to a number of different pathways following paternal exposure to environmental insult could potentially lead to an altered sperm sncRNA profile. As sperm harboured within the epididymal lumen are transcriptionally and translationally silent their sncRNA profile is curated through interactions with extracellular vesicles (EVs) produced by the epididymal epithelium. Hence, cellular responses to environmental or lifestyle factors that alter key members of **a)** miRNA transcriptional regulation, **b)** miRNA biogenesis, **c)** miRNA sorting and packaging into EVs and / or **d)** biogenesis, release and uptake of EV by recipient sperm would feasibly lead to the delivery of an altered sncRNA cargo to sperm and modulation to the sperm sncRNA profile of mature spermatozoa. This figure was generated with images from biorender.com

Admittedly, as the understanding of processes such as *MIR* gene transcriptional control, EV biogenesis in the male reproductive tract and the mechanism for sncRNA packaging and trafficking to recipient cells evolves, additional, currently unknown avenues that could account for a change in the sperm miRNA profile will likely emerge.

A current limitation of the studies described in Chapter 2 is a lack of direct causal evidence linking acrylamide exposure-induced dysregulation to transcription factors (TFs) and the subsequent sperm miRNA changes. Nevertheless, given the potential for the design of therapeutic interventions based on ameliorating such responses, it would be of considerable value for future experiments to focus on confirming the mechanistic model proposed here (Fig 3). Such experiments could utilise the *in vitro* model characterised in Chapter 3. Towards this goal, we have conducted preliminary work towards establishing an *in vitro* exposure of acrylamide that mimics the outcomes of *in vivo* exposure using the mECap18 cell line. This work has revealed an increased abundance of two acrylamide responsive miRNAs following 4 h of acrylamide (10mM) exposure; a response that occurs independent of any significant impact on mECap18 cell vitality (Fig 2a-c). Following the confirmation that the identified TFs are also elevated in this cell line following acrylamide exposure, the utilisation of pharmacological inhibitors or siRNA knockdown approach and subsequent assessment of the miRNA abundance in secreted EVs would provide evidence to test our proposed model. An example of a TF inhibitor that could be utilised is the established glucocorticoid receptor (NR3C1) antagonist, RU486, which is approved for use in humans (Lefevre *et al.*, 2017; Zhang *et al.*, 2006).



**Figure 2: mECap18 cells respond to *in vitro* exposure to acrylamide.** **a** mECap18 cells were treated with 10mM acrylamide or control and cells were harvested at 2, 4 and 8 h at which time cell concentration was determined and represented normalised to control cells (**b**). **c** RNA was isolated from cells treated with acrylamide for 4 h and RT-qPCR was performed to determine the abundance level of three miRNAs. Experiments were performed in triplicate and data is represented as mean  $\pm$  SEM. The U6 snoRNA was utilised as an endogenous reference miRNA and RT-qPCR data is represented using the  $\Delta\Delta Ct$  method.

The caput epididymis holds specific functional characteristics including major remodelling of the sperm sncRNA profile (Nixon *et al.*, 2015). Indeed, in examining the composition of the sperm sncRNA profile, significant alteration occurs following transit of the caput epididymis, with these epididymal acquired sncRNAs recently implicated in early embryo developmental events (Conine *et al.*, 2018). We implicate this segment of the epididymis in altering the sperm sncRNA profile following acrylamide exposure, a finding consistent with other models of paternal insult (Chan *et al.*, 2020; Fennell *et al.*, 2020). Transcriptomic examination of the mouse epididymis, separated into the caput, corpus and cauda segments, revealed that the acrylamide responsive TFs are more abundantly expressed in the proximal (caput and corpus) segments of the epididymis compared to the distal cauda epididymis, supporting the role of the proximal epididymis in response to stress (Rinaldi *et al.*, 2020). Further, the majority of

these TFs are abundantly expressed in the human epididymis highlighting the potential for such a response to be conserved among species (Uhlén *et al.*, 2015).

### **The role of sperm-borne sncRNA after fertilisation**

It is now known that a diverse repertoire of sperm-borne sncRNA are delivered to the oocyte at fertilisation and thereafter contribute to early embryonic development and the transgenerational inheritance of phenotypic characteristics. This important contribution of sperm-borne sncRNA was originally uncovered via the utilisation of a germline-specific ablation of the DICER-encoding gene, the endonuclease responsible for miRNA, endo-siRNA and tRF production. This mouse knockout model produced sperm with a partially deficient sncRNA profile, which subsequently failed to support normal preimplantation embryo development; a consequence that could be rescued by supplementation with wild-type sperm RNA (Yuan *et al.*, 2016). More recent advances have uncovered that approximately 10% of the transcriptomic changes associated with parthenotes can be attributed to the absence of sperm RNA (Conine *et al.*, 2020). Considering the role of this non-genetic factor in shaping the development of the early embryo, it is perhaps not surprising that sperm that harbour an altered sncRNA profile as a consequence of paternal environmental or lifestyle challenges, impart a legacy of these insults on the ensuing embryo. Indeed, numerous microinjection studies have established causal links between mRNA expression in the early embryo and stress sensitive sncRNAs delivered by the fertilising spermatozoon (Chen *et al.*, 2016; Conine *et al.*, 2018; Rodgers *et al.*, 2015; Sharma *et al.*, 2016). In a similar context, our own findings allude to a putative association between the altered sperm miRNA profile resulting from acrylamide exposure and subsequent embryo gene dysregulation and reduced embryo survival. The strategy of utilising IVF to produce embryos for our transcriptomic analysis provided a number of advantages, including the delivery of physiological levels of sperm sncRNA, natural sperm selection, such as motility and zona-pellucida binding and discrimination against any impacts of seminal vesicle factors or female reproductive tract responses. It did not however afford us the opportunity to confirm miRNAs as the specific

causal factor linking this chain of responses. Rather, we envisage that additional microinjection studies (utilising either isolated sperm miRNAs or a cocktail of synthetic miRNAs) into naïve zygotes and subsequent transcriptomic analysis are needed to provide definitive evidence of the contribution of acrylamide responsive sperm miRNAs to the regulation of embryo gene expression. Further, subsequent embryo transfer of these microinjected embryos and tracking of embryo survival would be advantageous in building a case that miRNAs are responsible for directing the cellular response that culminates in embryo loss following epididymal acrylamide exposure (Katen *et al.*, 2017). Irrespective, the data presented in Chapter 2 provides compelling evidence for the role of acrylamide responsive miRNAs in regulating embryo gene expression and hence the impetus to pursue additional investigation.

Potentially of greatest interest is the role of sperm borne sncRNA in directing transgenerational epigenetic inheritance. The increasing appreciation that sperm epigenetic machinery can transmit information from one generation to the next, influenced by the paternal environment, has led to the resurrection of the idea that an organism's environment can induce phenotypic changes in the offspring. Indeed, several animal models have demonstrated phenotypic alterations passed from an exposed parent to the offspring; the transmission of which is mediated by epigenetic factors in the germline, including sncRNAs (Gapp *et al.*, 2014; Rodgers *et al.*, 2015). This link between experience altered sncRNA and an offspring phenotype has been established by the ability to reproduce paternally induced offspring phenotypes via the microinjection of zygotes with RNA isolated from the spermatozoa of exposed animals (Chen *et al.*, 2016; Gapp *et al.*, 2014; Rodgers *et al.*, 2015). Further dissection of the sncRNAs responsible for this phenomenon has implicated the miRNA and tRF subclasses (Chen *et al.*, 2016; Conine *et al.*, 2018). Owing to the reduced embryo survival associated with acute epididymal acrylamide exposure, we focused our investigation on the mechanistic basis of acrylamide induced miRNA changes (Katen *et al.*, 2017). However, acrylamide is a key environmental chemical hazard that is known to have multigenerational effects following exposure. Indeed, chronic exposure models closely mimicking that of

humans, have shown that the male offspring of exposed sires present with increased sperm DNA damage and testicular induction of CYP2E1 (the sole enzyme responsible for acrylamide metabolism), despite themselves having had no direct acrylamide exposure (Katen *et al.*, 2016; Nixon *et al.*, 2012). Such data indicates a male germline dependant mechanism of inheritance. Considering our data demonstrated the ability of acrylamide exposure to influence the sperm sncRNA profile (Chapter 2) and the emerging role of sperm-borne sncRNA in the inheritance of an offspring phenotype, it is tempting to speculate a role for this non-genetic sperm factor in directing transgenerational inheritance following chronic acrylamide exposure. However, this hypothesis awaits experimental validation.

A curious observation of this feature of transgenerational inheritance is the seemingly adaptive advantage exposed fathers confer to their offspring. This response has lead researchers to suggest that the purpose of such inheritance may be priming the offspring for the environment into which they are born; a phenomenon that is often observed in plants and invertebrate models (Galloway, 2005; Kovalchuk *et al.*, 2004; Rechavi *et al.*, 2011). Consequently, however, if the environment of the offspring differs to that of the paternal preconception environment, such ‘advantages’ appear to be detrimental to the offspring. Interestingly, preliminary evidence suggests this could be the case with paternal chronic acrylamide exposure. Sperm from offspring of exposed fathers harbour contrasting levels of DNA fragmentation, depending on their own exposure, with paternal exposure alone leading to increased sperm DNA fragmentation, while direct acrylamide exposure in offspring reduces this damage to control levels (Chambers, 2019). Importantly, drawing on the Dutch famine, the impact of a ‘mismatch’ in environments has also been established in humans (Roseboom *et al.*, 2006; Schulz, 2010). The studied cohort experienced food restriction during gestation that was restored shortly after birth. Numerous studies on these subjects have revealed an increase in disease susceptibility later in life, including effects on metabolism and cardiovascular health (Roseboom *et al.*, 2006; Roseboom *et al.*, 2000), a result substantiated by animal studies (Bispham *et al.*, 2003; Jones and Friedman, 1982). Interestingly, these

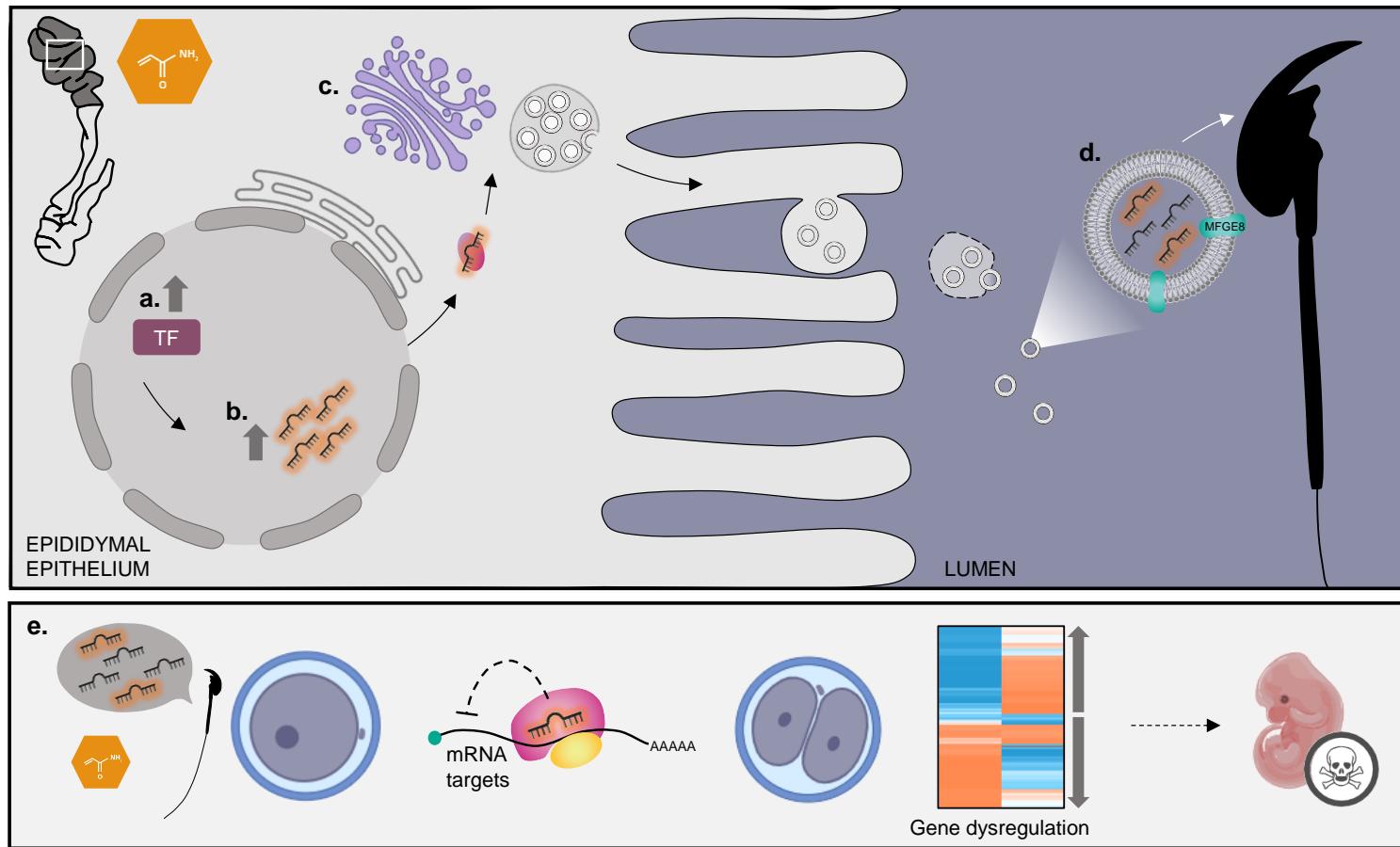
impacts are not apparent when food restriction was not confined to gestation (Stanner and Yudkin, 2001), further supporting the notion of parental experience priming the offspring for their environment. These studies highlight the importance of the preconception environment in subsequent offspring health and the need to understand the underlying mechanisms. For example, how experience-altered sperm sncRNAs, that direct transgenerational inheritance, act in the zygote to guide subsequent, complex developmental trajectories is yet to be resolved but remains imperative to understand epigenetic inheritance.

### **A key role of extracellular vesicles in sperm sncRNA modulation**

Epididymal extracellular vesicles are important actors in the acquisition and maintenance of sperm functions. Indeed, independent observations have established the transfer of a macromolecular payload from EVs to sperm *in vitro*, including protein, lipid and sncRNA cargo (Reilly *et al.*, 2016; Sharma *et al.*, 2016; Zhou *et al.*, 2019). Moreover, these vesicles display a comparable modification of their sncRNA cargo to that of sperm following exposure to environmental stress, a finding that we confirmed to be conserved in mice following acute acrylamide exposure (Chapter 2; (Chan *et al.*, 2020; Rompala *et al.*, 2018). As such, EVs are now accepted as fundamental conduits in shaping the sperm sncRNA profile, both during post testicular sperm maturation and in response to paternal exposure to environmental and lifestyle factors (James *et al.*, 2020; Trigg *et al.*, 2019). By way of example, microinjection of isolated sncRNA encapsulated in EVs sampled from the distal epididymis can rescue embryo lethality arising from ICSI with caput epididymal sperm (Conine *et al.*, 2018; Conine *et al.*, 2019). Moreover, a combination of co-incubation experiments and subsequent IVF and embryo transfer has identified the role of EVs, and their cargo, in mediating the transgenerational inheritance of phenotypes related to paternal stressors (Chan *et al.*, 2020; Rompala *et al.*, 2020). However, while EVs afford the encapsulated RNA molecules protection from degradation from RNases present in the extracellular environment and remain the leading candidate in moulding the sperm sncRNA profile, this does not discount the possibility that other delivery mechanisms may also contribute. Indeed, EV-mediated transfer does not

appear to account for the acquisition of sperm piRNAs during epididymal transit (Hutcheon *et al.*, 2017; Sharma *et al.*, 2018), and our data (Chapter 2), among others suggests EVs are not solely responsible for the trafficking of all sncRNA following stress (Rompala *et al.*, 2018). Alternate contributing mechanisms may include the delivery of RNA associated with specific binding proteins or harbouring specific modifications to provide the transcript sufficient stability to reach the transiting spermatozoa (Arroyo *et al.*, 2011; Wang *et al.*, 2010). Further, the recent discovery of long apical extensions from epididymal epithelial cells (referred to as ‘nanotubes’) that establish close interactions with spermatozoa, present a potentially novel mechanism of sncRNA transfer to sperm that warrants further investigation (Battistone *et al.*, 2020).

As key mediators of intercellular communication, EVs hold tremendous therapeutic opportunity, by harnessing their ability to deliver molecules, including sncRNA, to recipient cells. Notably, epididymal EVs are specific in their interaction, with surface orientated ligands likely guiding their selective targeting to sperm. This process is so finely tuned that there exist subpopulations of epididymal EVs that are capable of discriminating different populations of sperm (Frenette *et al.*, 2010). Our studies outlined in Chapter 3 have improved our understanding of the receptor-ligands involved in this interaction. The specificity of the sperm-EV interaction is attested by the retention of cargo transfer in 20% of the sperm population despite MGE8 perturbation, a finding that alludes to the presence of other ligands in guiding the specificity of this interaction. Identification of the EV ligands that direct this specificity will be imperative to harness an EV mediated form of therapeutic intervention to deliver sncRNA to spermatozoa. There remains a number of additional barriers to overcome before utilisation of EVs in such an intervention; many of which pertain to our limited understanding of the molecular basis of EV biogenesis in the epididymis and the process of sncRNA sorting that is responsible for directing sncRNA transcripts into EVs. Notably, our optimised *in vitro* model provides a suitable and tractable model to continue this promising avenue of research.



**Figure 3: A summary of the collective findings of this thesis and the proposed model of acrylamide induced sperm sncRNA alteration.**  
Upon acute acrylamide exposure, expression of key transcription factors (TFs) increase in epididymal epithelial cells of the proximal caput epididymis (**a**), which drives the transcription of *MIR* genes (**b**). After processing, these miRNAs appear destined for packaging into epididymal extracellular vesicles (EVs) (**c**) for delivery to spermatozoa, a process that is facilitated by the EV ligand milk fat globule-EGF factor 8 protein (MFGE8) and their recipient sperm receptors (**d**). This ultimately leads to a population of spermatozoa with an altered composition of sperm miRNAs that are delivered to the oocyte. Delivery of these altered sperm miRNAs subsequently leads to the dysregulation of gene expression, including mRNA targets of the altered sperm miRNA, in the 2-cell embryo (**e**) which may contribute to reduced levels of embryo survival. Figure was generated using biorender.com

### **Concluding Remarks:**

The main findings arising from this thesis are summarised in Figure 3, which serves to illustrate that acute acrylamide exposure elevates the expression of select TFs in caput epididymal epithelial cells, thereby driving the transcription of *MiR* genes. The resultant miRNAs appear destined for packaging into EVs and delivery to spermatozoa, in a process that is facilitated by MGE8 EV ligands and their recipient sperm receptors. Ultimately, this generates a population of spermatozoa with an altered composition of miRNAs that are delivered to the oocyte and influence gene expression in the early embryo. Although further studies are clearly needed to resolve the complex link between elevated TF expression and an altered sperm miRNA profile, the studies described in this thesis provide impetus to pursue such goals. This research serves to highlight the complex nature of the male germline response to environmental stressors and may aid the future goal of developing therapeutic interventions to restore the physiological sncRNA profile of spermatozoa following paternal stress and to inform public policy on the importance of preconception paternal health.

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