THE FUNCTION OF MOLECULAR CHAPERONES IN HUMAN SPERM-EGG RECOGNITION

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B Biotechnology (Honours Class 1)

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31st August, 2015

Declaration

Statement of Originality

The thesis contains no material which has been accepted 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 in the text. 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.

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

Thesis by Publication

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co- author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Signed

Elizabeth Grace Bromfield

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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 work in this thesis

1. Publications

Chapter 1: Introduction and literature review

Bromfield, E. G. and Nixon, B (2013). The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes. *Reproduction.* 145, R31–R42. DOI: 10.1530/REP-12-0316. Invited Review. **Published.**

Chapter 2:

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Chapter 3:

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Chapter 4:

Bromfield, E. G., Aitken, R. J. McLaughlin E.A. and Nixon, B (2015). HSPA2 forms a stable complex with Angiotensin Converting Enzyme (ACE) and Protein Disulfide Isomerase A6 (PDIA6) in human spermatozoa. *Molecular Human Reproduction.* **Accepted.**

Chapter 5: Final Discussion

Nixon, B., **Bromfield, E. G**., Dun, M. D., Redgrove, K. A., McLaughlin, E.A., Aitken, R. J. The role of the molecular chaperone heat shock protein A2 (HSPA2) in regulating human sperm-egg recognition. *Asian J Androl*.17, 268-573. DOI: 10.4103/1008-682X.151395. Invited Review, **Published.**

2. *Statements of contribution*

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

Brett Nixon
Date: 25/8/15

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3. Conference proceedings relevant to this thesis

Bromfield EG, Nixon B, Aitken RJ.
The effect of oxidative stress on sperm capacitation and the chaperone-mediated assembly of ZP receptor complexes on the human sperm surface. 17th annual biology RHD conference. Newcastle, Australia. November, 2012. *Oral presentation.*

Bromfield EG, Nixon B, Aitken RJ.
Impaired human sperm-zona pellucida receptor complex function in the presence of low levels of oxidative stress. International Conference of Andrology, Melbourne, Australia. February, 2013. ISSN: 2047-2919. *Poster presentation.*

Bromfield EG, Aitken, RJ, Redgrove KA, Nixon B.
Impaired human sperm-zona pellucida receptor complex function in the presence of low levels of oxidative stress. Abstract no. 36. 44th annual conference of the Society for Reproductive Biology. Sydney, Australia. August 2013. *Oral presentation* | Finalist in Oozoa award section.

Bromfield EG, Aitken, RJ, Nixon B.
Impaired human sperm-zona pellucida receptor complex function in the presence of low levels of oxidative stress. 18th annual Biology RHD conference. Newcastle, Australia. November, 2013. *Poster presentation* | winner of Best Poster prize.

Bromfield EG, Aitken RJ, Nixon B.
Regulation of HSPA2 function in human spermatozoa by cytotoxic lipid aldehydes. Abstract no. 28. The 12th International Symposium on Spermatology. August, 2014. *Oral presentation.*

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The impact of oxidative stress on chaperone-mediated human sperm-egg interaction. Abstract no.105. 45th annual conference of the Society for Reproductive Biology. Melbourne, Australia. August 2014. *Oral presentation.*

Bromfield EG, Aitken, RJ, Nixon B.
The impact of oxidative stress on chaperone-mediated human sperm-egg interaction. 3rd World Congress of Reproductive Biology. Edinburgh, Scotland. September 2014. *Poster presentation.*

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DeIuliis GN, **Bromfield EG**, Trigg N, Aitken RJ, Nixon, B.
Oxidative Stress-Induced Protein Modifications in Spermatozoa and Consequences for Sperm-Oocyte Recognition. 46th annual conference of the Society for Reproductive Biology. Adelaide, Australia. August 2015. *Oral presentation.*

4. Additional publications

1. **Bromfield EG**, Aitken RJ, Gibb Z, Lambourne SR, Nixon B.
Capacitation in the presence of methyl-β-cyclodextrin results in enhanced zona pellucida-binding ability of stallion spermatozoa. Reproduction. 2013 Dec 20;147 (2 Suppl):153-66. doi: 10.1530/REP-13-0393.

2. Dun MD, Anderson AL, **Bromfield EG**, Asquith KL, Emmett B, McLaughlin EA, Aitken RJ, Nixon B.
Investigation of the expression and functional significance of the novel mouse sperm protein, a disintegrin and metalloprotease with thrombospondin type 1 motifs number 10 (ADAMTS10). Asian Journal of Andrology, 2012 Aug; 35 (4):572-89. doi: 10.1111/j.1365-2605.2011.01235.x

5. *Awards*

Best Overall Oral Presentation | Australian Society for Medical Research Satellite Meeting | Newcastle 2015.

Best 3rd year Oral Presentation | 19th Annual Biology RHD conference | University of Newcastle November 2014

Finalist for Oozoa Award for best student presentation | Society for Reproductive Biology | 2012 & 2013

Society for Reproductive Biology Travel Award to attend the World Congress of Reproductive Biology | Scotland | 2014

Recipient of the FRRTC RHD conference scholarship | University of Newcastle | 2014

Best Student Poster Award |18th annual Biology RHD conference | University of Newcastle| November 2013

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Abstract

Sperm-egg recognition, adhesion and fusion are amongst the most fundamental interactions to occur between two cells in the body. As such, these processes are tightly regulated by numerous proteins adorning the surface of both the male and female gametes. However, given the importance of these proteins for fertilization success, even a slight deregulation of sperm-egg recognition machinery can have devastating effects on reproduction. This is evidenced by the distressingly common occurrence of sperm-egg recognition defects in the spermatozoa of male infertility patients. Unfortunately, as a majority of these men do not possess easily detectable defects in their spermatozoa, their infertility often goes undiagnosed with a large proportion of patients classified as having idiopathic infertility.

Recent work has shown that a deficiency in the molecular chaperone, Heat Shock Protein A2 (HSPA2) in the spermatozoa of these patients may be partly responsible for their inability to recognize the outer vestments of the egg, the zona pellucida (ZP). HSPA2 is known to be heavily involved in the remodeling of the sperm plasma membrane that occurs as these cells transcend the female reproductive tract. Moreover, our previous studies suggest that this protein regulates the assembly and presentation of important ZP-receptor protein complexes at the sperm surface that are required for interaction with the ZP. The studies within this thesis aimed to build upon this body of work by determining the molecular basis by which these cells lose the function and expression of HSPA2, and with it their ability to recognize the egg.

Herein, we provide the first evidence for a critical link between the attenuation of HSPA2 function and the presence of oxidative stress in human spermatozoa. By exploring this, we have revealed both the sensitivity of HSPA2 to oxidative modification by the lipid peroxidation product, 4-hydroxynonenal (4HNE) and an ensuing pathway that leads to the severe loss of ZP binding ability in human spermatozoa. Importantly, we have shown that the regulation of protein complex dynamics by HSPA2 and the perturbation of these events under conditions of oxidative stress extend to the novel HSPA2 client proteins, angiotensin converting enzyme (ACE) and protein disulfide isomerase A6 (PDIA6).

In accounting for the underrepresentation of HSPA2 in the patient population, we hypothesize that oxidative stress occurring in the developing germ cells of the testis, likely promotes similar modifications of HSPA2 by 4HNE but that these changes may result in more severe consequences for the stability of this protein. In the mouse testis, the proteolysis

of HSPA2 has been demonstrated in the absence of its stabilizing chaperone, BCL-2 associated athanogene 6 (BAG6). In this thesis we have demonstrated a stable interaction between HSPA2 and BAG6 in the testicular germ cells and mature spermatozoa of our own species, suggesting that a similar dependency on BAG6 may exist. Excitingly, our studies evaluating protein deficiency in the patient population have revealed that spermatozoa that lack the ability to interact with homologous human zonae pellucidae, associated with dysregulation of HSPA2 protein expression, also have a severe deficiency in BAG6 protein expression compared with fertile controls. These data lead us to propose that infertile spermatozoa are predisposed to a loss of HSPA2 protein expression through an absence of a BAG6-dependent protective mechanism against enzymes such as ubiquitin ligase that result in its degradation in testicular germ cells. This also provides impetus for further study into BAG6 as a potential molecular target for male factor infertility.

Taken together, the findings of this thesis contribute to our understanding of idiopathic male infertility by providing distinct links between oxidative stress and failed sperm-egg recognition. Importantly, this collection of studies offers a molecular understanding of the predictive value of HSPA2 in determining the fertilizing capacity of human spermatozoa. This in turn takes us closer to the development of HSPA2 as a positive molecular biomarker for sperm-egg binding competence, a crucial tool for the more accurate diagnosis of male factor infertility. Furthermore, our ability to recover a degree of sperm function through use of the nucleophile penicillamine, suggests that the deleterious effects of lipid aldehydes on HSPA2-mediated sperm-egg recognition may be ameliorated through antioxidant supplementation strategies. This gives credence to new practices that could improve the state of male reproductive health.

CHAPTER 1: LITERATURE REVIEW

The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes

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Chapter 1: Overview

The aim of the following review was to explore the dynamic role of molecular chaperones throughout the fertilization cascade. In this review we follow the function of chaperone proteins from their important role in the formation of ZP and hyaluronic acid binding sites on the maturing spermatozoon in the testis, through to their involvement in the formation and function of sperm-egg adhesion and fusion machinery at the surface of the male and female gametes.

A large suite of molecular chaperones have been implicated in the structural and functional maturation of mammalian spermatozoa within the testes and male reproductive tract. The presence of a number of these chaperones in mature spermatozoa raises the possibility that they fulfill important functions during the capacitation of these cells and in facilitating their interaction with the oocyte within the ampulla of the female reproductive tract. Consistent with this notion, molecular chaperones including CCT/TRiC, HSPA2, HSPA5, HSPD1, HSP90AA1, and HSP90B1 have been shown to be targets for capacitation-associated modification and/or have been indirectly implicated in ZP recognition. Specifically, chaperones such as these appear to assist in priming the sperm surface architecture for ZP interactions by virtue of their ability to assemble/present and/or activate ZP–receptor complexes. Although the role of chaperones in downstream interactions such as adhesion and fusion to the oolemma is less certain, a number of them including calreticulin, HSPA1A, HSPA5, HSP90AA1, and HSP90B1 have been localized to the surface of the oolemma and are thus well positioned to participate in these processes.

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The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes

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Abstract

The remarkable complexity of the molecular events governing adhesion and fusion of the male and female gametes is becoming apparent. Novel research suggests that these highly specific cellular interactions are facilitated by multiprotein complexes that are delivered to and/or assembled on the surface of the gametes by molecular chaperones in preparation for sperm–egg interaction. While the activation of these molecular chaperones and the mechanisms by which they shuttle proteins to the surface of the cell remain the subject of ongoing investigation, a compelling suggestion is that these processes are augmented by dynamic membrane microdomains or lipid rafts that migrate to the apical region of the sperm head after capacitation. Preliminary studies of the oocyte plasma membrane have also revealed the presence of lipid rafts comprising several molecular chaperones, raising the possibility that similar mechanisms may be involved in the activation of maternal fusion machinery and the regulation of oocyte plasma membrane integrity. Despite these findings, the analysis of oocyte surface multiprotein complexes is currently lacking. Further analyses of the intermediary proteins that facilitate the expression of key players in sperm–egg fusion are likely to deliver important insights into this unique event, which culminates in the cytoplasmic continuity of the male and female gametes.

Introduction

Originally identified as inducible proteins involved in the protection of cells from multiple stresses, molecular chaperones are now recognized as participants in a diverse range of functions due to their ability to selectively bind to hydrophobic residues of target proteins, directing their involvement in correct protein folding or degradation pathways (Ellis 1987, Hendrick & Hartl 1993). The heat shock proteins (HSPs), among other ubiquitous chaperone families, have well-documented roles in preventing the aberrant association or

aggregation of proteins in addition to facilitating protein synthesis, translocation, *de novo*folding, and higher ordered assembly of multiprotein complexes (Hendrick & Hartl 1993, Neuer *et al*. 2000). Among this multitude of functions, the ability of chaperones to mediate the assembly of oligomeric complexes is of particular interest as advances in functional proteomics have revealed that a significant portion of a cell's proteome realize their functional potential in multiprotein complexes rather than as singular entities (Sali *et al*. 2003).

This phenomenon is proving particularly true of the male gametes, which must complete several complex phases maturation in order to gain the functional competence to engage in one of the most intricate of all cellular interactions, fertilization. As transcriptionally inactive cells, spermatozoa rely heavily upon posttranslational modifications and remodeling of their constituent proteins to attain a state of functional maturity. For a sperm cell to interact with the oocyte, the activation of nascent, receptor-like proteins on the sperm surface is required, a process that has frequently been linked to the coordinated action of a subset of molecular chaperones (Ikawa *et al*. 2001, Asquith *et al*. 2004). Members of the HSP families, in addition to several other germ cell-specific chaperones, have been identified in both the mature spermatozoon of a number of species, including mouse (Asquith *et al*. 2004), human (Mitchell *et al*. 2007, Naaby-Hansen & Herr 2010), and pig (Spinaci *et al*. 2005), and in mature murine (Calvert *et al*. 2003) and bovine (Kawarsky & King 2001) oocytes and have been extensively implicated in gamete adhesion and fusion processes (Cayli *et al*. 2003, Asquith *et al*. 2004, Nixon *et al*. 2005, Huszar *et al*. 2007, Ikawa *et al*. 2011).

Preparation for oocyte interactions initially occurs through the formation of zona pellucida (ZP) binding sites during spermatogenesis (Huszar *et al*. 2007). Several molecular chaperones have been implicated in the remodeling of sperm membranes and the formation of oocyte binding

domains during the production of morphologically mature cells in the testes (Ikawa *et al*. 1997, Huszar *et al*. 2000). Though this prepares the spermatozoon for fertilization, additional phases of post-testicular maturation must be completed to attain a state of functional maturity. These events are initiated during their transit of the epididymis, wherein spermatozoa are bathed in a dynamic luminal microenvironment (Robaire *et al*. 2006, Cornwall 2009). Within this milieu, the sperm plasma membrane experiences the loss, gain, and modification of multiple proteins, a process that results in the attainment of the potential to move progressively and engage in oocyte interactions (Hermo *et al*. 1991). As discussed in the following sections, emerging evidence has implicated several HSP family chaperones in these modifications of the sperm plasma membrane during epididymal maturation (Lachance *et al*. 2010, Naaby-Hansen *et al*. 2010).

As a culmination of both testicular and post-testicular maturation, spermatozoa are able to reach a capacitated state as they progress through the female reproductive tract en route to the site of fertilization. As they ascend the female reproductive tract, the composition of sperm membrane proteins and lipids is subject to further dramatic alterations (Yanagimachi 1994, Harrison *et al*. 1996,Gadella & Harrison 2000, Gadella *et al*. 2008), resulting in the acquisition of the ability to recognize and bind to the outer vestments of the oocyte, the ZP. It is widely held that these binding events trigger acrosomal exocytosis and prime the cell for intercellular fusion with the oocyte. These collective events appear to be underpinned by a number of sperm surface protein complexes that comprise molecular chaperones, co-chaperones, and recognition proteins implicated in the specific interactions with oocyte ligands. Recently, a subset of these complexes has been demonstrated to undergo capacitation-associated assembly or activation (Asquith *et al*. 2005, Nixon *et al*. 2005). These chaperone-laden complexes may provide the basis for species-specific adhesion of spermatozoa to

homologous oocytes and thus have been the major focus of studies of molecular chaperones in this field. In the ensuing review, we seek to discuss the role of molecular chaperones in the mediation of sperm–oocyte adhesion and fusion, two germ cell-specific events that are coordinated largely through the formation of multiprotein complexes.

The role of chaperones in the acquisition of ZP binding potential

Although the expression of chaperones is traditionally viewed as being induced by heat shock and other stresses, members of the HSP60, HSP70, and HSP90 families have proven to be abundant components of the sperm surface that are constitutively expressed in the male germline and function as critical mediators of protein maturation during spermatogenesis (Miller *et al*. 1992, Boulanger *et al*. 1995). The generation of a mature spermatozoon requires multiple maturation steps; the first of these occurs in the testis and is assisted by the regulatory action of a diverse family of chaperones, including chaperonin-containing T-complex/TCP1-ring complex (CCT/TRiC), HSP60 (or HSPD1;Meinhardt *et al*. 1995), clusterin (Onoda & Djakiew 1990), HSPA2 (Huszar *et al*. 2000), and the testis-specific chaperones calmegin (CLGN; Ikawa *et al.* 1997, Ohsako *et al.* 1994), and calsperin (CALR3; Ikawa *et al.* 2011), which are developmentally regulated and expressed in spermatogenic cells (see also Dun *et al.* (2012*a*)).

The role of these chaperones extends to arguably the most essential step in the acquisition of sperm fertilizing potential during spermatogenesis, the remodeling of the sperm morphology through the extrusion of excess cytoplasm, and the formation of a number of highly specialized domains. This remodeling phase culminates in the formation of both hyaluronic acid and ZP binding sites on the sperm plasma membrane that are fundamental to the processes of cumulus oophorus penetration and ZP interaction (Huszar *et al*. 2000, 2007). Little is known regarding

the mechanisms that underpin ZP binding site formation; however, in immature human sperm, the presence of a low number of zona binding sites and hyaluronic acid receptor sites as well as a corresponding reduction in fertility potential has been causally related to the reduced expression of the 70 kDa testis-expressed chaperone, HSPA2 (Huszar *et al*. 2000, Celik-Ozenci *et al*. 2003).

In the human, HSPA2 is expressed in the synaptonemal complex of spermatocytes and is again synthesized during late spermiogenesis concurrently with cytoplasmic extrusion and sperm plasma membrane remodeling, processes that require major sperm protein translocations (Huszar *et al*. 2000). In concert with a number of other testis-expressed chaperones (see below), HSPA2 assists in the mediation of correct protein folding and translocation to appropriate subcellular domains to produce a morphologically mature spermatozoon (Huszar *et al*. 2000, 2003). The importance of HSPA2 in spermatogenic differentiation is reinforced by studies that indicate that the diminished expression of this chaperone is associated with apoptosis (Dix *et al*. 1996, Nasr-Esfahani *et al*. 2010), DNA fragmentation (Kovanci *et al*. 2001), a lack of histone–protamine substitution (Nasr-Esfahani *et al*. 2001), increased levels of lipid peroxidation (Huszar *et al*. 2000), increased frequency of chromosomal aneuploidies (Nasr-Esfahani *et al*. 2001), as well as IVF failure (Huszar *et al*. 1992, 2003). Indeed, in the mouse, the targeted elimination of HSPA2 leads to a complete arrest of spermatogenesis (Allen *et al*. 1996, Mori *et al*. 1997). These defects appear to be a result of misfolding, misexpression, or incorrect processing of proteins during spermatogenesis (Neuer *et al*. 2000).

In some instances, chaperones, such as the testis-specific calmegin, remain imperative to sperm function despite the fact that they are not expressed in the mature cell. Such chaperones are necessary as they ensure correct folding of endoplasmic reticulum glycoproteins that are

destined to play a more direct role in zona adhesion in addition to promoting their delivery to the appropriate plasma membrane domain (Ikawa *et al*. 1997). Indeed, disruption of the calmegin (Ikawa *et al*. 1997, Ikawa *et al*. 2001) and calsperin (Ikawa *et al*. 2011) genes in mice has been demonstrated to compromise male fertility due to the impaired formation of testis-specific t-fertilin (ADAM1A and ADAM2, where ADAM denotes a disintegrin and metalloprotease) and sperm surface s-fertilin (ADAM1B and ADAM2) complexes. Such defects are causally associated with impaired zona binding and reduced sperm transport through the uterotubal junction of the female reproductive tract (Ikawa *et al*. 2001). Interestingly, this phenotype is shared among several knockout models, including calmegin, calsperin, ADAM1A, and ADAM2, and appears to be attributed to the absence of ADAM3 from spermatozoa in each instance (Yamaguchi *et al*. 2006). Such a finding suggests that the assembly of the fertilin complexes by chaperones during spermatogenesis is essential for the formation of ZP recognition domains as well as the processing and/or escort of key receptor proteins such as ADAM3 (Muro & Okabe 2011).

Following their release from the testes, the second major phase of sperm maturation occurs as the cells progress through the dynamic environment of the epididymis, the extratesticular duct that connects the testis and the vas deferens. As a culmination of epididymal maturation, spermatozoa acquire the potential to move progressively and interact with the ZP. Chaperone proteins including clusterin (Hermo *et al*. 1991), HSPA5 (Lachance *et al*. 2010, Naaby-Hansen *et al*. 2010), and HSPD1 (Asquith *et al*. 2005) are thought to regulate epididymal maturation through their indirect roles in promoting the remodeling of sperm surface architecture (Dun *et al*. 2012*a*). Although the mechanisms by which proteins are incorporated into spermatozoa during epididymal maturation are yet to be completely resolved, it is postulated that this process occurs through sperm membrane fusion with small, epididymal

exosomal vesicles termed epididymosomes (Frenette & Sullivan 2001, Sullivan *et al*. 2007) and/or amorphous, electron-dense structures termed dense bodies (Asquith *et al*. 2005). The expression of several molecular chaperones in epididymosomes (Frenette & Sullivan 2001, Saez *et al*. 2003, Sullivan *et al*. 2005) and dense bodies, including HSPD1, suggests that these proteins may facilitate the bulk transfer of new protein onto the sperm surface during epididymal maturation (Asquith *et al*. 2005, Dun *et al*. 2011). In addition, there is some evidence to suggest a correlation between the presence of clusterin-positive sperm and bull fertility (Ibrahim *et al*. 2000).

In mature mouse sperm, HSPD1 has been shown to subsequently interact with additional chaperones HSPE1 and CCT/TRiC as well as a putative ZP receptor candidate ADAMTS10 (Walsh *et al*. 2008, Dun *et al*. 2011, 2012*b*), which exemplifies the important role this chaperone may play in epididymal sperm surface remodeling to prepare the cell for ZP interactions. In support of this model, HSPA5 has also been identified in epididymosomes (Lachance *et al*. 2010) and together with its putative client protein, ADAM7, is also transferred to the sperm surface during epididymal maturation (Oh *et al*. 2009). Within spermatozoa, these proteins form a multimeric complex (comprising an additional chaperone, calnexin, and integral membrane protein 2B), which has been implicated in sperm–oocyte interactions (Han *et al.* 2011). These findings draw interesting analogies with a number of other cell types, including that of metastatic cancers, where chaperones have been intimately tied to the function of metalloproteases, raising the possibility that this form of interaction may regulate a range of biological and pathological processes (Ikawa *et al*. 2010).

Despite the collective importance of these maturation stages in the acquisition of ZP binding potential, they do not represent the final changes necessary for the spermatozoon to engage in

fertilization. Rather, a further pivotal maturation phase must take place post-ejaculation within the female reproductive tract.

Multi-protein complexes in sperm capacitation and ZP interaction

Upon reaching the site of fertilization, the ampulla, spermatozoa must penetrate two barriers before fusing with the oocyte plasma membrane, or oolemma. The first of these is a hyaluronic acid-rich stratum of cumulus cells that surround the oocyte and the second is the extracellular matrix of the oocyte itself, the ZP (Hartmann *et al*. 1972). Despite the development of both ZP and hyaluronic acid binding sites during spermatogenesis and the acquisition of binding potential as spermatozoa transit the epididymis, these cells require a distinct period of residence within the female reproductive tract before they are able to successfully partake in such interactions (Austin 1951, Chang 1951). The collective changes that spermatozoa undergo within this environment, termed capacitation, enable the cells to respond to signals arising from the cumulus oocyte complex and complete a process of acrosomal exocytosis, rendering them competent for fusion with the oolemma.

The ZP is comprised of a suite of sulfoglycoproteins, namely ZP1, ZP2 and ZP3, that are highly conserved in most mammalian species (though an additional ligand, ZP4/B, has been reported in human and pig oocytes) (Wassarman *et al*. 1999, Lefievre *et al*. 2002, Yonezawa *et al*. 2012). It is generally held that these ligands govern sperm binding in most species (see also Reid *et al*. (2011)). Remarkably, however, various models are currently still under consideration regarding the identity of the primary sperm receptor within the ZP and the mechanisms by which spermatozoa adhere to this matrix (see also Visconti & Florman (2010)). Similarly, investigations into the identity of the corresponding sperm surface receptor(s) that recognize the appropriate ligand(s) on the ZP have also failed to provide definitive answers.

Indeed, there is a growing literature of murine knockouts of auspicious receptor protein candidates (including β-1,4-galactosyltransferase (GALT1), arylsulfatase A (ARSA), and sperm adhesion molecule 1 (SPAM1); for a complete list, see Ikawa *et al*. (2010)) that each fail to result in complete infertility (Hess *et al*. 1996, Asano *et al*. 1997,Baba *et al*. 2002). Rather, various degrees of reduced binding capability are exhibited, raising the possibility that this process encompasses a degree of functional redundancy and that a number of sperm proteins act in concert to mediate ZP adhesion. The coordination of the activity of these proteins to ensure productive ZP interactions is thus emerging as an important research focus.

In most eutherian mammals, sperm capacitation is thought to be initiated by the activation of a cAMP-mediated pathway that culminates in the tyrosine phosphorylation of multiple sperm proteins (Visconti *et al*. 1995*a*, 1995*b*, Leclerc*et al*. 1996). Molecular chaperones feature prominently among this suite of proteins, with HSP90AA1, HSP90B1 and HSPD1 being among the proteins revealed to display tyrosine phosphorylation as a consequence of capacitation (Ecroyd *et al*. 2003, Asquith *et al*. 2004). Current models suggest that the phosphorylation of these chaperones during capacitation triggers their active role in the assembly of ZP recognition proteins into complexes and/or the translocation of these complexes to the surface of spermatozoa in preparation for fertilization (Ecroyd *et al*. 2003, Asquith *et al*. 2004, Nixon *et al*. 2005,Gadella 2008). Further to this indirect role in gamete adhesion, sperm surface chaperones also have purported functions as adhesion molecules that mediate the recognition of sulfoglycolipids during gamete binding (Boulanger *et al*. 1995,Mamelak & Lingwood 2001).

Recently, the technique of blue native PAGE (BN-PAGE), which was originally developed for the analysis of electron transport chain multienzyme complexes (Schägger & von Jagow

1991, Schägger *et al*. 1994), has been adapted for the assessment of multimeric sperm surface complexes in mice and humans (Dun *et al*. 2011, Redgrove *et al*. 2011). This technique allows for the electrophoretic resolution of native protein complexes that retain their biological activity. In human and mouse spermatozoa, the use of BN-PAGE in parallel with Far-Western blotting with whole solubilized zonae has revealed several primary multiprotein complexes that possess affinity for homologous ZP (Dun *et al*. 2011, Redgrove *et al*. 2011).

One such complex has been reported to comprise the protein components of the CCT/TRiC complex (CCT1–CCT8), a double-ring structure that functions as a molecular chaperone with a key role in regulating the formation of multiprotein complexes (Feldman *et al*. 1999, Guenther *et al*. 2002). Putative evidence in the form of co-immunoprecipitation, co-localization, and proximity ligation assays has identified ZP binding protein 2 (ZPBP2) as one of the most compelling client proteins for the CCT/TRiC complex in mature spermatozoa (Dun *et al*. 2011,Redgrove *et al*. 2011). Originally implicated in secondary ZP binding, a more recent study has shown that male mice null for ZPBP2 are subfertile and display defects in ZP interaction and penetration (Lin *et al*. 2007). In mice, there is additional evidence that certain CCT/TRiC complex subunits are translocated to the sperm surface during sperm capacitation (Dun *et al*. 2011).

Another prominent class of chaperones that has been identified on the sperm surface and implicated in the regulation of ZP interactions is the HSP70 family (Naaby-Hansen *et al*. 2010). As with the CCT/TRiC complex, HSP70 family chaperones also have well-documented roles in the facilitation of both transmembrane protein transport and assembly of stable protein complexes (Mayer & Bukau 2005). One member of the HSP70 family that displays exclusive (mouse) or predominant (human) expression in the testes appears to be essential for male

fertility. Indeed, aberrant expression of this chaperone, HSPA2, has been correlated with a phenotype of severe male factor infertility in humans, specifically affecting the ability of spermatozoa to interact with homologous oocytes *in vitro* (Eddy 1999, Huszar *et al*. 2007). In both mice and humans, HSPA2 has a fundamental role in spermatogenesis, with targeted deletion of the protein in the former species leading to an early arrest of this process and a concomitant absence of spermatozoa (Eddy 1999). In humans, the expression levels of HSPA2 have been positively correlated with the success of fertilization*in vitro* (Huszar *et al*. 2000, 2006, Cayli *et al*. 2003) and hence are purportedly able to predict the fertility status of men with a high degree of accuracy (Ergur *et al*. 2002).

Characterization of HSPA2 in our own laboratory has revealed that this chaperone is present in the acrosomal domain of human spermatozoa and is a component of at least five high-molecular-mass protein complexes (Redgrove *et al*. 2012), including a subset of those shown previously to possess ZP affinity (Redgrove *et al*. 2011). Consistent with these data, we have secured evidence that the most dominant of the HSPA2 complexes contains two additional proteins, both of which have been previously implicated in sperm–zona interactions (Redgrove *et al*. 2012). Furthermore, in agreement with the published results of Huszar *et al*., we have been able to demonstrate a significant reduction in HSPA2 levels in the spermatozoa of men with isolated lesions in their ability to engage in interactions with ZP of homologous oocytes *in vitro* (Redgrove *et al*. 2012). Our current work is focusing on whether the deficit in ZP adhesion either results from aberrant formation of ZP binding sites in the early stages of spermiogenesis (Huszar *et al*. 2000) or may be the result of the inability of HSPA2 to participate in sperm surface remodeling events during capacitation such as facilitating the assembly and/or presentation of ZP receptors on the sperm surface in preparation for ZP interaction.

In addition to our own work on the assembly of sperm surface complexes, Han *et al*. have independently identified an alternative chaperone-laden multiprotein complex on the surface of mouse spermatozoa. Interestingly, as documented above, this complex, comprising HSPA5, calnexin, integral membrane protein 2B, and ADAM7, is apparently assembled during capacitation (Han *et al*. 2011). While the function of this complex has yet to be fully elucidated, the expression of ADAM7 has been linked to the presence of additional ADAM proteins, ADAM2 and ADAM3 (Kim *et al*. 2006), that are important for adhesion of spermatozoa to the ZP (Muro & Okabe 2011). In addition, it is known that HSPA5 is involved in promoting adhesion of high-quality spermatozoa to oviductal epithelial cells (OEC) in the isthmus of the female reproductive tract. The formation of this reservoir is believed to have pro-survival effects in terms of maintaining sperm in a non-capacitated, quiescent state in preparation for the oocyte to be released to the ampulla (Topfer-Petersen *et al*. 2002). Interestingly, the chaperones HSPD1 and HSPA5 have also been localized to the surface of bovine OEC and have thus been implicated in sperm–OEC binding (Boilard *et al*. 2004).

Also consistent with our own work, the complex identified by Han *et al*. was shown to reside in membrane microdomains or lipid rafts, specialized regions of the membrane that provide a platform for the functional assembly and presentation of multiprotein complexes (Stein *et al*. 2006, Nixon *et al*. 2009,Han *et al*. 2011). The partitioning of chaperone complexes into the raft environment has also been observed for HSPA2 in human spermatozoa (Nixon*et al*. 2011) and for components of the CCT/TRiC complex in mouse spermatozoa (Dun *et al*. 2011). These membrane domains also comprise a number of additional putative ZP receptor proteins, including GALT1, ZP3R, and SPAM1, reinforcing their role in the remodeling of the sperm surface and in ZP binding (Fig. 1; Nixon *et al*. 2009, Asano *et al*. 2010). The mechanism(s) by

CHAPTER 2:

The impact of oxidative stress on chaperone-mediated human sperm-egg interaction

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Chapter 2: Overview

The objective of this manuscript was to explore a mechanism by which sperm-egg recognition may be compromised in human spermatozoa. Given the prevalence of oxidative lesions in the spermatozoa of male infertility patients, this study evaluated the sensitivity of the HSPA2 chaperone to oxidative damage and in doing so established a link between sperm-egg recognition and cellular oxidative stress.

These studies were performed by inducing oxidative stress *in vitro* through the treatment of normal spermatozoa with low levels of hydrogen peroxide (H_2O_2) or the cytotoxic lipid aldehyde 4-hydroxynonenal (4HNE) prior to capacitation. Without having a significant impact on capacitation-associated changes in membrane fluidity or protein tyrosine phosphorylation, the oxidative treatments employed in this study severely disrupted the ability of human spermatozoa to interact with homologous ZP. This reduction in sperm-egg recognition was associated with the impaired surface expression of a zona pellucida-receptor complex comprising HSPA2, SPAM1 and ARSA. Upon investigating the mechanism of action, it was demonstrated that oxidative stress targeted HSPA2 for covalent modification by 4HNE. These data raise the possibility that 4HNE modification of HSPA2 may cause attenuation of its chaperone activity and a subsequent dysregulation of its ability to remodel the sperm surface in preparation for ZP binding. In support of this model, the use of penicillamine to reduce the bioavailability of 4HNE enabled a partial recovery of ARSA surface expression and ZP adherence in H_2O_2 treated spermatozoa.

The data presented in this chapter strongly suggest that even low levels of oxidative stress may result in a deregulation of crucial cell recognition machinery. Given that diminished HSPA2 expression in the sperm of infertile men has been causally linked to a loss of zona pellucida binding ability, the implications of this work may extend to an increase in understanding of idiopathic male factor infertility. Certainly, this study suggests that the chaperone HSPA2 is sensitive to the effects of oxidative stress, highlighting an important direction for further study into a causative link between non-enzymatic post-translational modifications and a loss of the functional presence of HSPA2 in cases of male infertility.

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The impact of oxidative stress on chaperone-mediated human sperm–egg interaction

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STUDY QUESTION: How does oxidative stress impact upon human sperm–egg interaction and in particular the formation of zona pellucida-receptor complexes on the sperm surface?

SUMMARY ANSWER: Oxidative stress during human sperm capacitation resulted in the chemical alkylation of the molecular chaperone heat shock protein A2 (HSPA2), a concomitant reduction in surface expression of the zona pellucida-receptor arylsulphatase A (ARSA) and a severe loss of zona pellucida binding ability.

WHAT IS KNOWN ALREADY: An inability to bind to the zona pellucida is commonly encountered in the defective spermatozoa generated by male infertility patients; however, the underlying mechanisms remain unresolved. Recent studies have revealed that zona pellucida binding is mediated by molecular chaperones, particularly HSPA2, that facilitate the formation of multimeric zona pellucida-receptor complexes on the surface of mammalian spermatozoa during capacitation.

STUDY DESIGN, SIZE, DURATION: Spermatozoa were collected from healthy normozoospermic donors ($n = 15$). Low levels of oxidative stress were induced in populations of non-capacitated spermatozoa by a 1 h treatment with 4-hydroxynonenal (4HNE) or hydrogen peroxide (H_2O_2) and then these insults were removed and cells were capacitated for 3 h.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Motility, membrane fluidity, protein tyrosine phosphorylation and lipid raft distribution were evaluated after sperm capacitation to determine the impact of oxidative stress on this process. The surface expression of ARSA and sperm adhesion molecule 1 (SPAM1) was observed using fluorescence microscopy, and the ability of treated cells to interact with homologous human zonae pellucidae was assessed through gamete co-incubation. Proximity ligation was used to evaluate the state of the HSPA2-laden zona pellucida-receptor complex and an immunoprecipitation approach was taken to establish the chemical alkylation of HSPA2 by the cytotoxic lipid aldehyde 4HNE. The validity of these findings was then tested through treatment of oxidatively stressed cells with the nucleophile penicillamine in order to scavenge lipid aldehydes and limit their ability to interact with HSPA2. All experiments were performed on samples pooled from two or more donors per replicate, with a minimum of three replicates.

MAIN RESULTS AND THE ROLE OF CHANCE: The oxidative treatments employed in this study did not influence sperm motility or capacitation-associated changes in membrane fluidity, tyrosine phosphorylation and lipid raft redistribution. However, they did significantly impair zona pellucida binding compared with the capacitated control ($P < 0.01$). The reduction in zona pellucida binding was associated with the impaired surface expression ($P < 0.02$) of a zona pellucida-receptor complex comprising HSPA2, SPAM1 and ARSA. Proximity ligation and immunoprecipitation assays demonstrated that impaired zona pellucida binding was, in turn, associated with the chemical alkylation of HSPA2 with 4HNE and the concomitant disruption of this zona pellucida-receptor complex. The use of penicillamine enabled a partial recovery of ARSA surface expression and zona pellucida adherence in H_2O_2-treated cells. These data suggest that the ability of low levels of oxidative stress to disrupt sperm function is mediated by the production of lipid aldehydes as a consequence of lipid peroxidation and their adduction to the molecular chaperone HSPA2 that is responsible for co-ordinating the assembly of functional zona pellucida-receptor complexes during sperm capacitation.

LIMITATIONS, REASONS FOR CAUTION: While these results extend only to one particular zona pellucida-receptor complex, we postulate that oxidative stress may more broadly impact upon sperm surface architecture. In this light, further study is required to assess the impact of oxidative stress on additional HSPA2-laden protein complexes.

WIDER IMPLICATIONS OF THE FINDINGS: These findings link low levels of oxidative stress to a severe loss of sperm function. In doing so, this work suggests a potential cause of male infertility pertaining to a loss of zona pellucida recognition ability and will contribute to the more accurate diagnosis and treatment of such conditions.

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Introduction

The generation of reactive oxygen species (ROS) by mammalian spermatozoa is crucial for the activation of several physiological processes (Aitken *et al.*, 1998; de Lamirande and Lamothe, 2009; Aitken and Curry, 2011). Not least of these is capacitation, a complex and tightly regulated series of transformations that spermatozoa must undergo in the female reproductive tract in order to interact with an oocyte and carry out fertilization (de Lamirande and Gagnon, 1993). While sperm capacitation is characterized by its key outcomes (hyperactivated motility, sperm–zona pellucida recognition and an acquired ability to undergo acrosomal exocytosis), it is also coupled with a suite of important cellular events that make it possible to monitor this process *in vitro*. These events include an influx of calcium and bicarbonate (Gadella and Harrison, 2000; Florman *et al.*, 2008), increases in membrane fluidity (Davis *et al.*, 1979), a redistribution of membrane rafts (Boerke *et al.*, 2008; Nixon *et al.*, 2009), rises in both intracellular pH (Vrendenburgh-Wilberg and Parrish, 1995; Aitken *et al.*, 1998) and cAMP (Visconti *et al.*, 1995a; Tardif *et al.*, 2004) and the activation of numerous signalling cascades that underpin key protein phosphorylation events (Visconti *et al.*, 1995b; O'Flaherty *et al.*, 2003, 2005). Importantly, the stimulation and regulation of a number of these events by both exogenous and endogenous ROS is now well recognized in a number of species, including the human (de Lamirande and Gagnon, 1995; Aitken *et al.*, 2003; O'Flaherty *et al.*, 2006), mouse (Herrero *et al.*, 2003) and the bovine (O'Flaherty *et al.*, 2003).

Despite their fundamental importance for sperm capacitation, levels of ROS that exceed physiological relevance can stimulate a cascade of events leading to oxidative DNA damage and eventually an apoptotic-like death (Aitken, 2011). Additionally, peroxidative damage to the sperm plasma membrane caused by oxidative stress can result in an irreparable loss of sperm function and long-term infertility (Aitken *et al.*, 1991, *2012a*). Moreover, oxidative stress to spermatozoa is a particular risk in the *in vitro* systems that are used for assisted conception, as removal of seminal plasma during sperm preparation leaves these cells particularly susceptible to oxidative attack (Aitken and Clarkson, 1998).

Although sperm capacitation events have been intently studied over the past decade, an understanding of some of the key molecular mechanisms that underpin this process is still lacking (Aitken and Nixon, 2013). A notable example is that despite decades of work towards the identification of a single sperm surface receptor that negotiates adhesion and binding to the zona pellucida, murine knockouts of putative zona pellucida receptors have generally failed to elicit complete infertility (as reviewed by Reid *et al.*, 2011). However, these seemingly confounding studies have given rise to a novel concept that molecular chaperones may be involved in the capacitation-dependent assembly of zona-receptor complexes on the sperm surface during capacitation (Asquith *et al.*, 2004; as reviewed by Bromfield and Nixon, 2013). In support of this proposal, recent work in our laboratory has identified a number of high molecular weight, multimeric protein complexes on the surface of human spermatozoa that show affinity for homologous zonae pellucidae (Redgrove *et al.*, 2011, 2012). The most dominant of these complexes has been extensively characterized and comprises three key proteins that form a stable interaction in human spermatozoa, namely sperm adhesion molecule 1 (SPAM1, previously PH20), which is a hyaluronidase implicated in both cumulus cell matrix dispersal and sperm–zona pellucida binding (Lathrop *et al.*, 1990; Kimura *et al.*, 2009), Arylsulphatase A (ARSA), which has previously been implicated in sperm–egg adhesion and binding (Carmona *et al.*, 2002; Tantibhedhyangkul *et al.*, 2002) and a molecular chaperone of the heat shock protein 70 family, heat shock protein A2 (HSPA2), which has been the subject of studies both in our laboratory and independently, as a key marker of both sperm maturity and zona pellucida binding competence (Ergur *et al.*, 2002; Huszar *et al.*, 2007; Redgrove *et al.*, 2012). Our collective evidence suggests that HSPA2 coordinates a capacitation-associated rearrangement of this complex (Redgrove *et al.*, 2012). Thus, the hyaluronidase SPAM1 is present on the surface of non-capacitated sperm to aid in their penetration of the hyaluronic acid-rich matrix of cumulus cells surrounding the oocyte. Thereafter, capacitated sperm present ARSA on their surface to assist in their initial tethering to the zona pellucida via its interaction with the sulphated regions of glycans adorning the surface of the zona pellucida (Fig. 1).

Failure to bind to the zona pellucida is a commonly encountered attribute of defective spermatozoa within the subpopulation of patients exhibiting male infertility (Bastiaan *et al.*, 2002; Liu *et al.*, 2004). In light of the above findings, we postulate that the clinical disruption of sperm–zona pellucida binding may be facilitated by defects in the HSPA2-mediated assembly of zona-receptor complexes on the sperm surface during capacitation. In some cases, we have found that this involves an actual loss of the HSPA2 chaperone from the sperm proteome (Redgrove *et al.*, 2012, 2013). However, we also propose that the same phenotype would result if the functionality of HSPA2 were somehow compromised. Since oxidative stress is a common feature of male infertility, we undertook the present study to determine whether this form of stress can inhibit the ability of human spermatozoa to bind to the zona pellucida and, if so, whether this loss of functionality is associated with a failure to assemble and present HSPA2-mediated zona-receptor complexes on the sperm surface.

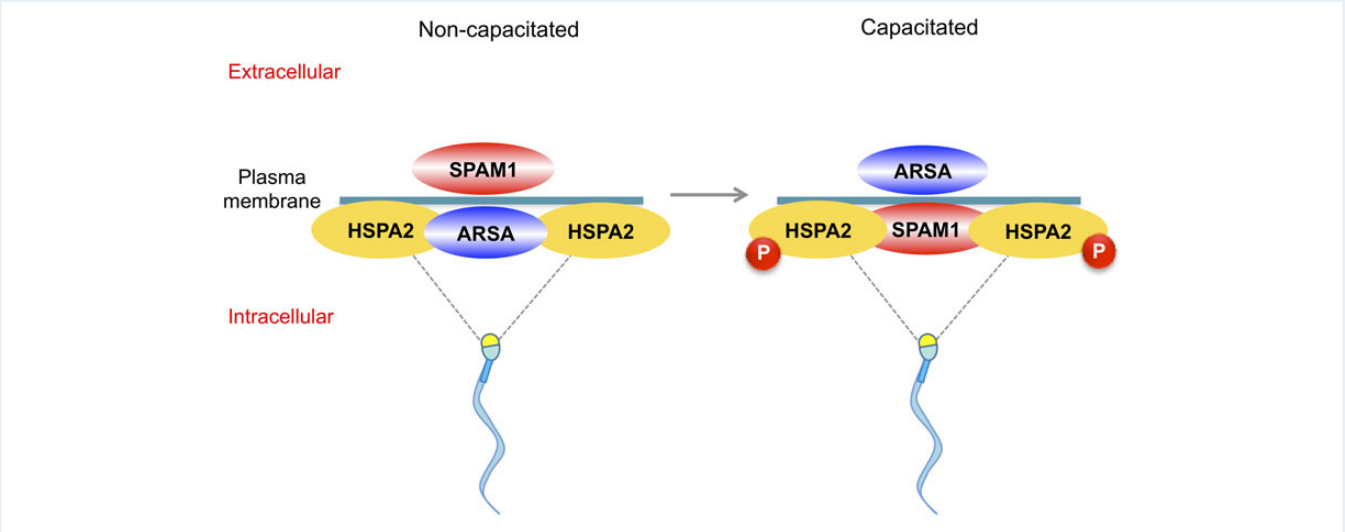

Figure 1 The coordination of SPAM1 and ARSA surface expression during capacitation by the molecular chaperone HSPA2. Our collective evidence suggests that the activation of HSPA2 during capacitation coordinates a capacitation-associated rearrangement of a protein complex comprising sperm adhesion molecule 1 (SPAM1) and arylsulphatase A (ARSA) (Redgrove *et al.*, 2012, 2013). Thus, the hyaluronidase SPAM1 is present on the surface of non-capacitated sperm to aid in their penetration of the hyaluronic acid-rich matrix of cumulus cells surrounding the oocyte. Thereafter, capacitated sperm present ARSA on their surface to assist in their initial tethering to the zona pellucida via its interaction with the sulphated regions of glycans adorning the surface of the zona pellucida. *P* = protein tyrosine phosphorylation.

Materials and Methods

Reagents

Unless specified, chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of research grade. The following conjugated antibodies were purchased to characterize proteins of interest: anti-phosphotyrosine [PT66-FITC (fluorescein isothiocyanate)] anti-phosphotyrosine [PT66-HRP (horse-radish peroxidase)]. Albumin, ammonium persulphate and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) were obtained from Research Organics (Cleveland, OH, USA); D-glucose, sodium hydrogen carbonate, sodium chloride, potassium chloride, calcium chloride, potassium orthophosphate and magnesium sulphate were all of analytical reagent grade, purchased from Merck (BDH Merck, Kilsyth, VIC, Australia). Tris was from ICN Biochemicals (Castle Hill, NSW, Australia), and Percoll from GE Healthcare (Rydalmere, NSW, Australia). Nitrocellulose was from GE Healthcare (Buckinghamshire, UK) while highly pure Coomassie Brilliant Blue G250 was obtained from Serva (Heidelberg, Germany). SYTOX green cell vitality stain was purchased from Invitrogen (Carlsbad, CA, USA). Mowiol 4-88 was from Calbiochem (La Jolla, CA, USA), and paraformaldehyde was supplied by ProSciTech (Thuringowa, Australia). A rabbit polyclonal antibody to HSPA2 was purchased from Sigma-Aldrich (Cat # HPA000798) along with a rabbit monoclonal antibody to ARSA (Cat # HPA005554). A mouse monoclonal antibody to SPAM1 was purchased from Abnova (Cat # H00006677-A01, Taipei City, Taiwan) and a mouse, monoclonal antibody to CD59 was purchased from Abcam (Cat # ab9183). Anti-4HNE rabbit polyclonal antibody (Cat # HNE11-S) was purchased from Jomar diagnostics (Stepney, SA, Australia). Appropriate HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma-Aldrich.

Ethical approval

The experiments described in this study were conducted using pooled human semen samples obtained with informed written consent from a panel of 15 healthy normozoospermic donors (the majority of proven fertility) in accordance with the University of Newcastle's Human Ethics Committee guidelines (Approval No. H-2013-0319).

Preparation of human spermatozoa

Enrichment of human spermatozoa was achieved using 45 and 90% discontinuous Percoll gradients as described previously (Nixon *et al.*, 2011). High-quality spermatozoa were recovered from the base of the 90% Percoll fraction and resuspended in a bicarbonate-free non-capacitating (NC) form of Biggers, Whitten and Whittingham medium (NC BWW; Biggers *et al.*, 1979) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin and 20 mM HEPES buffer and 1 mg/ml polyvinyl alcohol (PVA, osmolarity of 300 mOsm/kg). The cells were then pelleted by centrifugation at 500 × *g* for a further 15 min and resuspended at a concentration of 10×10^6 cells/ml. All experiments were performed on samples pooled from two or more donors per replicate with a minimum of three replicates examined.

Induction of oxidative stress in human spermatozoa

Oxidative stress was induced in populations of non-capacitated human spermatozoa through treatments with either 4-hydroxynonenal (4HNE) (Sigma) or hydrogen peroxide (H_2O_2) (Sigma) at concentrations of 50, 100 and 150 μM. Both 4HNE and H_2O_2 were chosen to induce oxidative stress based on their efficacy established in previous studies (Aitken *et al.*, 2011). Cells at a concentration of 10×10^6 cells/ml were resuspended in 4HNE or H_2O_2 and then incubated for 1 h at 37°C. Treated spermatozoa were then washed once in NC BWW. Following treatment, cell motility assessments were conducted at 37°C with an HTM-IVOS CASA system (Hamilton-Thorne Biosciences, version 12.3) using chamber slides of 20 μm depth (Leja, GN Nieuw-Vennep, The Netherlands). HTM-IVOS settings were adjusted as follows for human spermatozoa: negative phase

contrast optics, recording rate 60 frames/s, minimum contrast 80, minimum cell size 3 pixels, low size gate 1.0, high size gate 2.9, low intensity gate 0.6, high intensity gate 1.4, non-motile head size 6, non-motile head intensity 160, progressive VAP (average path velocity) threshold value, 25 μm/s, slow cells VAP cut-off, 5 μm/s, slow cells VSL (straight line velocity) cut-off, 11 μm/s and threshold STR (straightness) >80%. Progressive cells were those exhibiting a VAP of >25 μm/s and a STR of >80%. Approximately eight fields were assessed in order to generate data on an average of 207 (range 168–277) spermatozoa per sample. The following sperm motility parameters were analysed: total motility (%); forward progressive motility (%); amplitude of lateral head displacement (ALH; μm); track speed (VCL; μm/s). Additionally, the percentage of hyperactivated spermatozoa (of 200 cells) was assessed by examining the spermatozoa using phase contrast optics at room temperature. Cell vitality was assessed using an eosin vitality stain as previously described (Aitken *et al.*, 2012a).

Capacitation of human spermatozoa

To induce capacitation *in vitro*, human spermatozoa were resuspended in a capacitating (CAP) form of BWW composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin, 20 mM HEPES buffer and 1 mg/ml PVA (osmolarity of 300 mOsm/kg) and supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic adenosine monophosphate (dbcAMP). Cells were incubated in this medium for 3 h at 37°C under an atmosphere of 5% CO_2: 95% air with cells resuspended at a concentration of 10×10^6 cells/ml. Non-capacitated cells were incubated for the same period of time (without exposure to CO_2) in BWW prepared without $NaHCO_3$ (NC BWW). Following incubation, cell motility, hyperactivation and vitality were assessed, as described above. Populations of spermatozoa were then used for the assays outlined below.

Assessment of membrane fluidity and membrane raft localization

Merocyanine 540 (Sigma) was used to assess membrane fluidity of human spermatozoa. Following capacitation, aliquots of human spermatozoa from each treatment were diluted to 1×10^6 cells/ml and incubated in SYTOX green vitality stain at 37°C for 10 min. This preparation was washed once in BWW and then incubated in 2.7 μM merocyanine 540 at 37°C for 10 min. Preparations were then washed once in BWW and 200 cells from each treatment were scored on a Zeiss fluorescence microscope at excitation wavelengths 590 nm (merocyanine 540) and 470 nm (SYTOX green) (Carl Zeiss, Thornwood, NY, USA). Merocyanine positive sperm were identified through bright red fluorescence over the entire head and the absence of SYTOX green staining.

The localization of membrane raft marker, G_{M1} ganglioside, was visualized in human spermatozoa by staining with Alexa Fluor 555-labelled B subunit of cholera toxin (CTB) as previously described (Nixon *et al.*, 2011). For each treatment, 200 cells were classified into two fluorescent patterns (head and tail or head only labelling).

Analysis of protein tyrosine phosphorylation

Sodium dodecyl sulphate polyacrylamide gel electrophoresis/western blotting

Following treatment, human spermatozoa were pelleted via centrifugation and resuspended for protein extraction as previously described (Reid *et al.*, 2012). Protein extracts were then boiled in the presence of NuPAGE LDS sample buffer (Invitrogen) containing 8% β-Mercaptoethanol, subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 4–12% Bis–Tris gels (Invitrogen) and then electrotransferred to nitrocellulose membranes using conventional western blotting techniques (Towbin *et al.*, 1979). To detect proteins of interest, membranes were blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST, pH 7.4) then probed with HRP-conjugated anti-phosphotyrosine antibody (α-PT66) diluted 1:10 000 in TBST supplemented with 1% BSA under constant rotation for 1 h at room temperature. Membranes were washed in TBST (3 × 10 min) and labelled proteins were visualized using an enhanced chemiluminescence detection kit (ECL plus, Amersham Bioscience) according to the manufacturer's instructions.

Immunocytochemistry

Following capacitation, spermatozoa were fixed in 4% paraformaldehyde, washed 3× with 0.05 M glycine in phosphate-buffered saline (PBS) and then applied to poly-L-lysine-coated glass coverslips. Cells were permeabilized with 0.2% Triton X-100, then placed in a humid chamber and blocked in 3% BSA/PBS for 1 h. Coverslips were then washed in PBS and incubated in FITC-conjugated α-PT66 antibody diluted 1:100 with 1% BSA/PBS for 1 h at 37°C. Following this, coverslips were washed (3 × 5 min) in PBS before mounting in 10% mowiol 4–88 (Calbiochem) with 30% glycerol in 0.2 M Tris (pH 8.5) and 2.5% 1,4-diazabicyclo-(2.2.2)-octane (DABCO). Samples were examined with a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss Pty, Sydney, Australia), and human spermatozoa were classified as phosphotyrosine (PT66) positive when displaying uniform fluorescence across the full length of the sperm flagella, as previously described (Urner and Sakkas, 2003).

Zona pellucida binding assessment

Human zonae pellucidae were obtained from IVF Australia clinics (Human Ethics Approval No. H-2013-0134) and stored in a high salt storage medium consisting of 1.5 M $MgCl_2$, 0.1% dextran, 0.01 mM HEPES buffer and 0.1% PVA at 4°C. Prior to use, zona-intact oocytes were washed 3× in PBS and four oocytes per treatment were placed in droplets of BWW under water-saturated mineral oil at 37°C in an atmosphere of 5% CO_2: 95% air and allowed to equilibrate for 30 min. The spermatozoa were diluted to a concentration of 1×10^6 cells/ml and 20 μl of sperm suspension was added to each droplet of oocytes. Gametes were co-incubated under the same conditions for a further 30 min. Oocytes were then washed 3× by serial aspiration through droplets of BWW to remove any unbound sperm. After washing in BWW, oocytes were mounted on glass slides under coverslips suspended at each corner by 80% paraffin wax and 20% Vaseline gel and the number of motile sperm bound to each zona pellucida was assessed using phase contrast microscopy (Zeiss Axioplan 2).

Agonist-induced acrosome reaction

To induce acrosomal exocytosis, human spermatozoa were incubated for 30 min with 0.5% v/v dimethylsulfoxide (DMSO) vehicle control or 2.5 μM calcium ionophore (A23187). Sperm were then incubated in prewarmed hypo-osmotic swelling media (HOS; 0.07% w/v sodium citrate; 1.3% w/v fructose) for a further 30 min at 37°C. Sperm preparations were washed in PBS, placed on poly-L-lysine-coated slides and allowed to air dry. The cells were then permeabilized in ice-cold methanol and subjected to immunocytochemistry with TRITC-labelled peanut agglutinin lectin (PNA, 1:200), as previously described (Redgrove *et al.*, 2013). The acrosomal status of human spermatozoa was assessed with a Zeiss LSM510 laser scanning confocal microscope. Acrosome reacted cells were identified by the appearance of a curled tail and either the complete absence of PNA staining over the acrosomal region or the restriction of this labelling to the equatorial segment of the sperm head (Rathi *et al*, 2001).

Surface labelling of human spermatozoa

Non-capacitated, 4HNE-treated and H_2O_2-treated sperm suspensions were incubated with primary antibody (Anti-SPAM1, Anti-ARSA or

Anti-CD59; diluted 1:100) for 1 h. The cells were subsequently washed 2× with BWW and incubated with FITC-conjugated secondary antibody (diluted 1:500) for a further 30 min. Following three washes with BWW, the cells were incubated with 20 mg/ml of propidium iodide (PI) for 1 min and assessed for surface fluorescence using a Zeiss fluorescence microscope ($t = 0$ min). Following capacitation of untreated, 4HNE-treated and H_2O_2-treated spermatozoa, fresh sperm suspensions were incubated with primary antibody for 1 h, washed in BWW (×2) and incubated in corresponding FITC-conjugated secondary antibodies for 30 min. After three washes, these cells were also incubated in PI and immediately assessed for surface fluorescence ($t = 180$ min). Antibodies against CD59 were used as a positive control for assessment of protein expression on the surface of human spermatozoa, as previously described (Redgrove *et al.*, 2012), and the proportion of sperm expressing ARSA and SPAM1 on their surface was recorded for 200 cells from each treatment.

Blue Native PAGE

Both H_2O_2-treated and capacitated spermatozoa were prepared for blue native PAGE (BN-PAGE) as previously described (Redgrove *et al.*, 2013). Briefly, cell pellets were resuspended in native lysis buffer consisting of 1% *n*-dodecyl b-D-maltoside, 0.5% Coomassie Blue G250 and a cocktail of protease inhibitors (Roche, Mannheim, Germany) and incubated at 4°C on an orbital rocker for 30 min. Samples were centrifuged for 20 min at 14 000 × *g* and then dialysed against Blue Native cathode buffer (purchased from Invitrogen, Carlsbad, CA, USA) overnight at 4°C. Dialysed native protein lysates were loaded onto blue native polyacrylamide gels (NativePAGE Novex 4–16%, Bis–Tris gels; Invitrogen) and resolved using a NativePAGE cathode and anode buffer (Redgrove *et al.*, 2011) and run at 4°C at 100 V for the duration of the stacking gel layer and at 200 V for the resolving gel layer for ~3 h. Following retrieval from the cassettes, gels were either stained with Coomassie G250 or prepared for western blotting with an antibody to HSPA2.

Proximity ligation assay

Duolink *in situ* proximity ligation assays (PLAs) were conducted in accordance with the manufacturers' instructions on fixed human spermatozoa adhered to poly-L-lysine-coated coverslips (OLINK Biosciences, Uppsala, Sweden). Samples were blocked in Duolink blocking solution and then incubated with primary antibodies (anti-SPAM1, anti-ARSA, anti-HSPA2 and anti-tubulin) overnight at 4°C. Oligonucleotide-conjugated secondary antibodies (PLA probes) were then applied for 1 h at 37°C and ligation of the PLA probes was performed, and the signal amplified according to the manufacturer's instructions. The fluorescent signal generated when molecules are in close association (<40 nm) was visualized using fluorescence microscopy and could be quantified by recording the proportion of 100 spermatozoa displaying a collection of red fluorescent dots over the sperm head. Specificity of this reaction was ensured by performing proximity ligation with antibodies to the target antigens combined with anti-tubulin antibodies with which they should not interact.

Immunoprecipitation

Capacitated and 4HNE-treated spermatozoa were prepared as above. Cell lysis was performed on populations of ~100 × 10^6 cells from each treatment at 4°C for 2 h in lysis buffer consisting of 10 mM CHAPS, 10 mM HEPES, 137 mM NaCl and 10% glycerol with the addition of protease inhibitors (Roche). Lysis was completed with a centrifugation step performed at 14 000 × *g* at 4°C for 20 min. The cell lysates were then added to 50 µl aliquots of washed protein G Dynabeads and incubated under rotation to preclear at 4°C for 1 h. Anti-HSPA2 antibody at a concentration of 10 µg in 200 µl of PBS was conjugated to fresh aliquots of washed (supernatant removed) Dynabeads by incubation for 2 h at 4°C under rotation. Following antibody binding the cross-linking reagent, 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP), was added at a final concentration of 2 mM and cross-linking was performed at room temperature for 30 min after which 20 mM TRIS was added to each tube for an additional 15 min at room temperature to quench the reaction. Beads were washed (3×) in 200 µl of lysis buffer and the wash supernatants were kept and stored at −20°C.

Immunoprecipitation was then performed by adding 1 ml precleared lysate to HSPA2 antibody-bound beads and incubating under rotation overnight at 4°C. After incubation, supernatant was transferred to a clean tube and washed (3×) in 200 µl of PBS. Finally, beads were resuspended in 100 µl of PBS and transferred to a fresh tube to avoid co-elution of proteins bound to the tube.

Target antigen was eluted from the beads by boiling in the presence of SDS-loading buffer containing 8% β-mercaptoethanol. The same elution was performed on precleared beads and these solutions were loaded onto a NuSep 4–20% Tris–glycine gel for analysis via SDS–PAGE. In addition, bead-only and antibody-only controls were prepared by loading 10 µl of protein-G bead slurry and 5 µl of anti-HSPA2 in the presence of SDS-loading buffer into appropriate gel lanes. The third wash of the antibody-bound beads for each treatment was also loaded onto the gel after boiling in the presence of SDS-loading buffer. A duplicate gel was also prepared for immunoblotting and both were resolved at 150 V for ~1 h. Electro-transfer of proteins was performed as previously described (Redgrove *et al.*, 2013). To detect proteins of interest, membranes were blocked in 3% w/v BSA in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST, pH 7.4) then probed with anti-HSPA2 antibody diluted 1:1000 in TBST supplemented with 1% BSA under constant rotation overnight at 4°C. Membranes were washed in TBST (3 × 10 min) and then incubated in anti-rabbit HRP-conjugated secondary antibody (diluted 1:1000) for 1 h at room temperature. Following three washes in TBST, proteins were visualized using an enhanced chemiluminescence detection kit (ECL plus, Amersham Bioscience) according to the manufacturer's instructions. Following this, membranes were stripped for 30 min at room temperature in ReProbe solution (G Biosciences, St. Louis, MO, USA), blocked in 5% w/v skim milk in TBST and probed with anti-4HNE antibody diluted 1:1000 in TBST supplemented with 3% skim milk overnight at 4°C. After three washes, membranes were incubated in anti-rabbit HRP-conjugated secondary antibody (1:1000) for 1 h at room temperature before being visualized as above.

Penicillamine treatment

To investigate the effect of aldehydes on sperm function, the nucleophile D-penicillamine was used to covalently bind aldehydes, thereby limiting their bioavailability, as described previously (Aitken *et al.*, 2012a). Briefly, human spermatozoa were prepared via Percoll centrifugation and then exposed to 50 µM H_2O_2 for 1 h at 37°C (as above). Following this treatment, cells were washed once in BWW and then capacitated for 3 h in CAP BWW supplemented with either 0.5 or 1 mM D-penicillamine (Sigma). Following capacitation, spermatozoa were washed in BWW and either processed for assessment of zona pellucida binding, surface labelling with α-ARSA antibodies, or co-immunoprecipitation using the protocols described above.

Statistical analysis

All experiments were replicated at least three times with independent samples and data are expressed as mean values ± SE. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test using Microsoft Excel (Version 14.0.0; Redmond, Washington, DC, USA). Differences were considered significant for $P < 0.05$.

Results

Sperm motility and viability after treatment with 4HNE and H_2O_2

To establish the concentrations of 4HNE and H_2O_2 to be used for this series of experiments, a dose-dependent study was conducted to

evaluate both sperm motility and viability following treatment with each agent. Concentrations of 50, 100 and 150 μM were trialled based on previous studies (Aitken *et al.*, 2011, *2012a*).

As expected, dose-dependent decreases in both viability and motility were recorded after treatment with all concentrations of 4HNE or H_2O_2 (Fig. 2). Despite this, at the lowest concentration of each agent (50 μM), motility remained at 65 and 62% for 4HNE and H_2O_2, respectively. For the purpose of this study, only low levels of oxidative stress were desirable to evaluate subtle differences in sperm capacitation and zona pellucida binding ability without explicitly compromising the ability of these cells to function. For this reason, concentrations of 50 μM of each agent were chosen for subsequent analyses. These concentrations of 4HNE and H_2O_2 are well within the range attained under conditions of oxidative stress, which in the case of 4HNE can reach 5 mM (Uchida, 2003) while in the case of H_2O_2, a concentration of 50 μM is within the range found in many biological fluids (Halliwell *et al.*, 2000).

For the purpose of the next series of experiments, spermatozoa that were treated with 50 μM 4HNE or 50 μM H_2O_2 were then washed free of these agents and capacitated for 3 h in BWW supplemented with 3 mM pentoxifylline and 5 mM dbcAMP.

Assessment of capacitation status after treatment with 4HNE and H_2O_2

During the initial phase of capacitation in the female reproductive tract, mammalian spermatozoa experience a rapid loss of membrane sterols in response to increased levels of bicarbonate and calcium in the extracellular environment (Flesch *et al.*, 2001). This loss of membrane sterols promotes a degree of membrane fluidization through a 'scrambling' of the lipid components of the plasma membrane (Boerke *et al.*, 2008). *In vitro*, this capacitation-driven membrane fluidization can be evaluated using a fluorescent probe, Merocyanine 540, which is incorporated into spermatozoa that have a degree of membrane destabilization (Flesch *et al.*, 2001). This probe was therefore used in our study to quantify the number of sperm that were able to undergo this capacitation-dependent process after treatment with 50 μM 4HNE or H_2O_2 (Fig. 3A).

Unexpectedly, the percentage of sperm that were positive for Merocyanine 540 after treatment with either ROS generating agent was statistically similar ($P \geq 0.05$) to the percentage of spermatozoa exhibiting a positive merocyanine response under standard capacitating conditions. Interestingly, the percentage of spermatozoa that displayed evidence of membrane fluidization was greater having undergone pretreatment with 4HNE (87.5%) or H_2O_2 (74.5%) than the capacitated control (CAP; 54%). Notably, all treatments showed a statistically significant ($P \leq 0.05$) increase in the proportion of spermatozoa displaying a positive merocyanine response compared with the non-capacitating control devoid of bicarbonate (NC; 18%).

An additional correlate of the capacitation process that has been documented in human spermatozoa (Nixon *et al.*, 2011) is the redistribution of membrane rafts from a uniform distribution throughout both the sperm head and flagella in non-capacitated cells to a discernible aggregation within the apical region of the sperm head, following capacitation (Fig. 3B). This capacitation-dependent shift in raft distribution is thought to be permitted by the fluidization of the plasma membrane and can be monitored using fluorescently labelled choleratoxin B (CTB) that has affinity for the G_{M1} ganglioside structural components

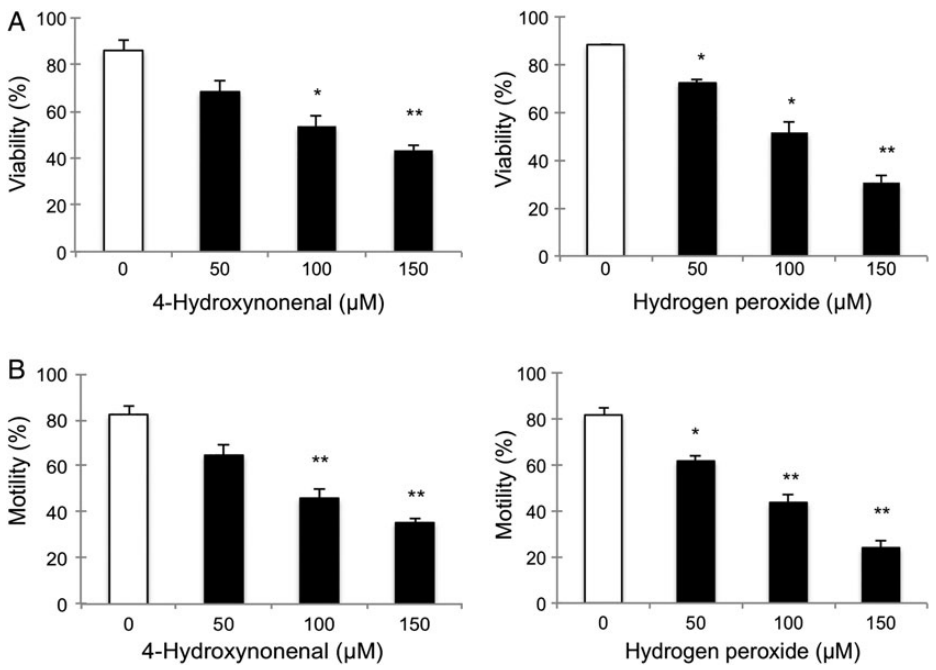

Figure 2 Effect of oxidative stress on human sperm motility and viability. Non-capacitated human spermatozoa were treated with either 50, 100 or 150 μM 4HNE or H_2O_2 for 1 h and then capacitated in BWW supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic AMP. A dose-dependent decrease in motility was observed using a HTM-IVOS CASA system and a dose-dependent reduction in sperm viability was observed through use of an Eosin vitality stain. Statistical analyses were performed using a Student's *t*-test, $^*P < 0.05$; $^{**}P < 0.01$.

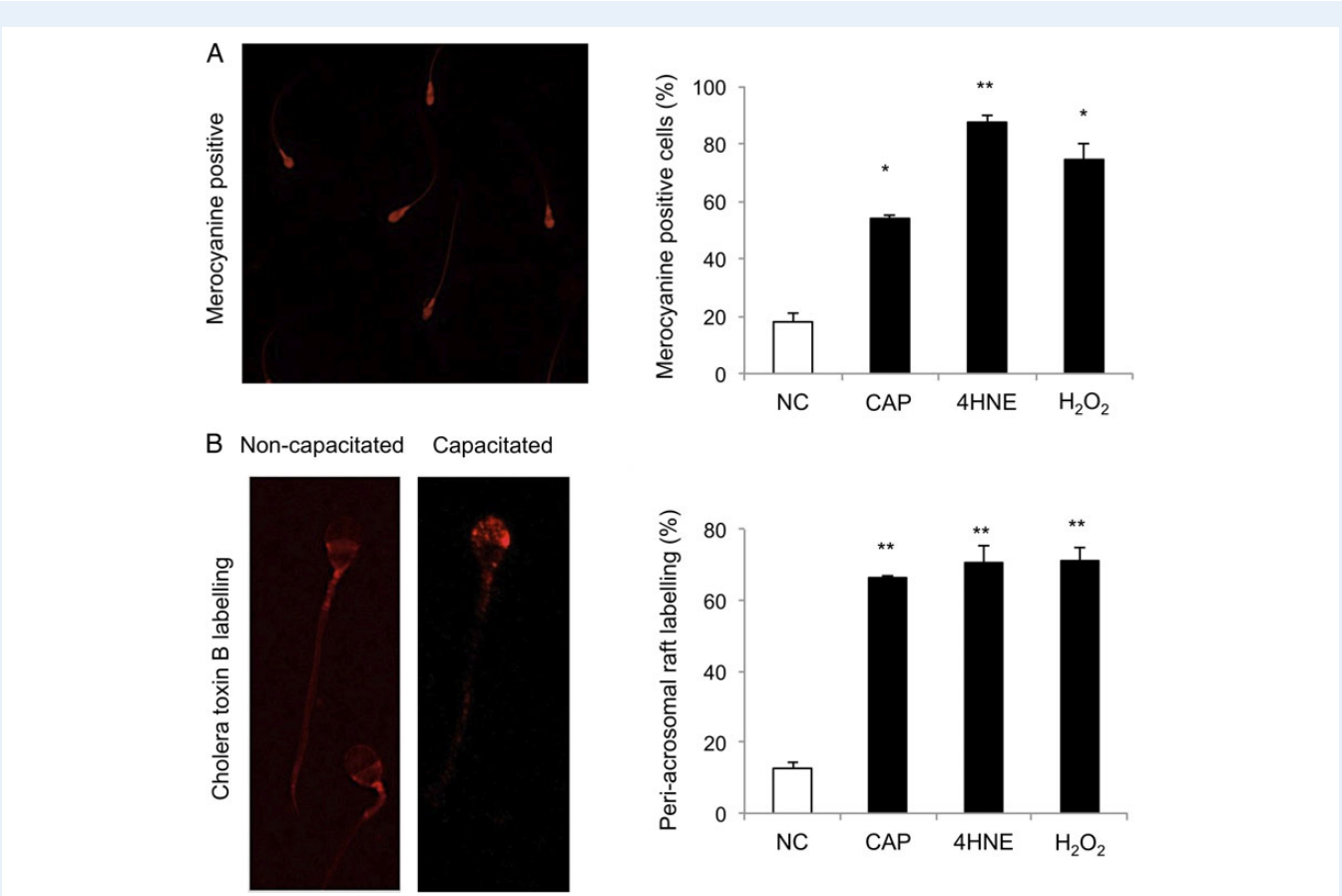

Figure 3 Assessment of human sperm membrane fluidization and membrane raft localization in response to oxidative stress. To assess the capacitation state of human spermatozoa pretreated with 4HNE or H_2O_2 prior to capacitation, merocyanine 540 was selected to monitor membrane destabilization and fluidization and fluorescently labelled CTB was used to localize the G_{M1} gangliosides of membrane rafts. Spermatozoa treated with 4HNE or H_2O_2 were washed free of these agents and then capacitated for 3 h in BWW supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic AMP. Live cells were then either stained with Merocyanine 540/SYTOX green or incubated in Alexa Fluor 555-labelled CTB and fixed with 4% paraformaldehyde. Non-capacitated (NC), capacitated (CAP), 4HNE-treated (4HNE) and H_2O_2-treated (H_2O_2) cells were assessed for membrane fluidity by the recording the proportion of 200 viable cells displaying a Merocyanine positive signal across three biological replicates (red fluorescence over the sperm head), images were taken on a fluorescence microscope using a × 40 objective (**A**) and spermatozoa were assessed for raft localization by scoring 200 cells across three biological replicates displaying the peri-acrosomal labelling pattern typical of a capacitated cell. Images were taken on a fluorescence microscope using a × 100 objective (**B**). Statistical analyses were performed using a Student's *t*-test, $^*P < 0.05$; $^{**}P < 0.01$.

of membrane rafts (Nixon *et al.*, 2010, 2011). Using this marker, the number of human spermatozoa displaying the CTB labelling pattern typical of CAP cells was recorded after incubation under NC or CAP conditions and after treatment with 4HNE and H_2O_2. Consistent with the results observed for membrane fluidization, in an environment of enhanced oxidative stress a similar percentage of human spermatozoa displayed the restricted apical CTB labelling pattern after treatment with 4HNE (71%) and H_2O_2 (71%) compared with that observed under CAP conditions (66.5%). In contrast, only 13% of sperm incubated under NC conditions displayed any evidence of membrane raft redistribution to the sperm head.

To further explore these findings, the ability of human spermatozoa to undergo protein tyrosine phosphorylation after exposure to oxidative stress was evaluated. Through immunocytochemistry, only 8% of cells incubated under NC conditions displayed typical PT66 positive labelling of the whole flagella. This was in contrast to CAP spermatozoa that presented evidence of complete flagellar protein tyrosine phosphorylation in 74% of the population. When these cells were treated with either 4HNE or H_2O_2 prior to capacitation, no significant difference in the percentage of cells displaying complete PT66 labelling was observed compared with the capacitated control with 75 and 65% of cells from these treatments, respectively, displaying labelling patterns typical of a capacitated cell (Fig. 4A). These results were consistent with western blotting analyses (Fig. 4B), which revealed a similar profile and overall level of phosphotyrosine expression following treatment with either 4HNE or H_2O_2 (Fig. 4B).

Collectively, this evaluation of membrane fluidization, raft reorganization and capacitation-induced protein tyrosine phosphorylation suggests that human spermatozoa treated with low levels of 4HNE and H_2O_2 remain capable of undergoing the changes necessary to achieve the early stages of capacitation, *in vitro*. However, one of the most dynamic properties acquired by capacitating sperm is the ability to recognize and bind to the zona pellucida (Dun *et al.*, 2010). To explore the impact of low levels of oxidative stress on the functional end-points of sperm capacitation, the remaining experiments of this study evaluated

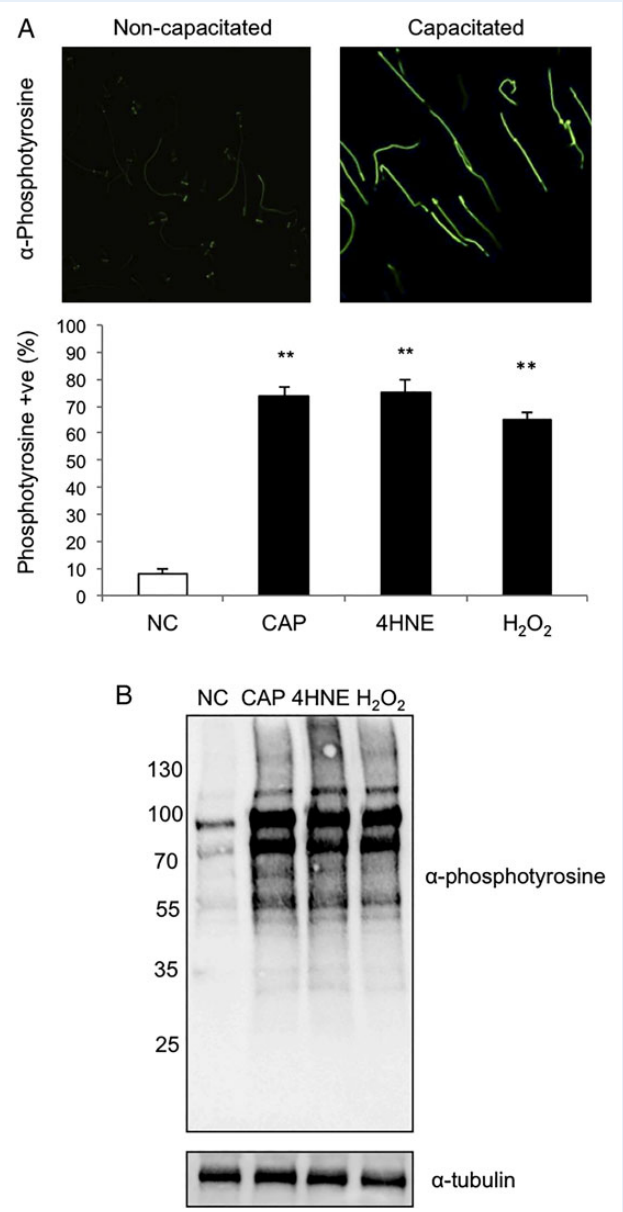

Figure 4 Impact of 4HNE and H_2O_2 on tyrosine phosphorylation of human sperm proteins. Aliquots of non-capacitated (NC) and capacitated spermatozoa (CAP), and capacitated spermatozoa pretreated with 4HNE or H_2O_2 were (**A**) fixed in 4% paraformaldehyde and incubated with a FITC-conjugated antibody to phosphotyrosine (α-PT66). Cells were scored as phosphotyrosine positive if they displayed complete flagella labelling, scoring a total of 200 cells from each treatment over three biological replicates. Images were taken on a confocal microscope using a ×40 objective. (**B**) Cells were lysed and the proteins prepared for immunoblotting with a HRP-conjugated anti-phosphotyrosine antibody (α-PT66). Following development, blots were stripped and re-probed with anti-α-tubulin to ensure equivalent protein loading. This experiment was repeated three times and representative immunoblots are shown. Statistical analyses were performed using a Student's *t*-test, $^{**}P < 0.01$.

the ability of 4HNE- and H_2O_2-treated human spermatozoa to engage in interactions with the zona pellucida.

Evaluation of the zona pellucida binding ability of human sperm after treatment with 4HNE and H_2O_2

To examine the impact of 4HNE and H_2O_2 on the zona pellucida binding ability of human spermatozoa, a binding assay incorporating human ova was used (Fig. 5A). As expected, a significantly greater number of CAP human sperm were capable of interacting with the zona pellucida when compared with those incubated under NC conditions ($P < 0.001$) with an average of 33 sperm bound/oocyte after capacitation and an average of only two sperm bound/oocyte after a 3 h incubation in media devoid of bicarbonate (7% of CAP control). Despite having comparable levels of motility and number of hyperactivated cells within the samples (Supplementary data, Table SI), human spermatozoa treated with 4HNE and H_2O_2 prior to capacitation were unable to bind tightly to the zona pellucida with an average of only seven spermatozoa bound/oocyte for 4HNE-treated cells (22% of CAP control) and two spermatozoa bound/oocyte following H_2O_2 treatment (7% of CAP control). These numbers were significantly lower than those achieved under the same capacitating conditions without prior exposure to 4HNE and H_2O_2 ($P < 0.01$). Treatment with H_2O_2, in particular, reduced the binding ability to a level statistically similar to that achieved under NC conditions ($P > 0.05$). While not the focus of these studies, treatment with 4HNE and H_2O_2 also had a significant impact on the ability of human spermatozoa to undergo an agonist-induced acrosome reaction, with a 2-fold decrease in the number of acrosome reacted spermatozoa detected in populations exposed to such insults prior to capacitation (Supplementary data, Fig. S1).

The impact of oxidative stress on the co-ordinated surface expression of SPAM1 and ARSA during capacitation

Given the important role of ARSA in mediating contact between the sperm surface and the sulphated residues of zona pellucida glycans, a reduction in expression of this receptor on the surface of capacitated human sperm might be expected to have deleterious consequences on the binding ability of these cells during gamete co-incubation.

This study revealed that ~78% of NC cells (untreated, $t = 0$) were surface labelled with anti-SPAM1 compared with only ~17% with anti-ARSA (Fig. 5B). However, incubation in CAP media led to a significant decrease in the surface expression of SPAM1 such that only 34% of the viable sperm population was positively labelled. In contrast, ARSA surface expression showed a dramatic increase after capacitation, resulting in ~62% of the viable population being labelled after 180 min, a result that reflects flow cytometry data published by Redgrove *et al.* (2013). However, the capacitation-dependent increase in ARSA surface expression was significantly compromised by pretreatment of the cells with reagents capable of inducing oxidative stress. In this context, only 21 and ~10% of live spermatozoa exhibited surface expression of ARSA following 4HNE and H_2O_2 treatments, respectively (Fig. 5B). Importantly, CD59 was detected on the surface of >80% of spermatozoa across all

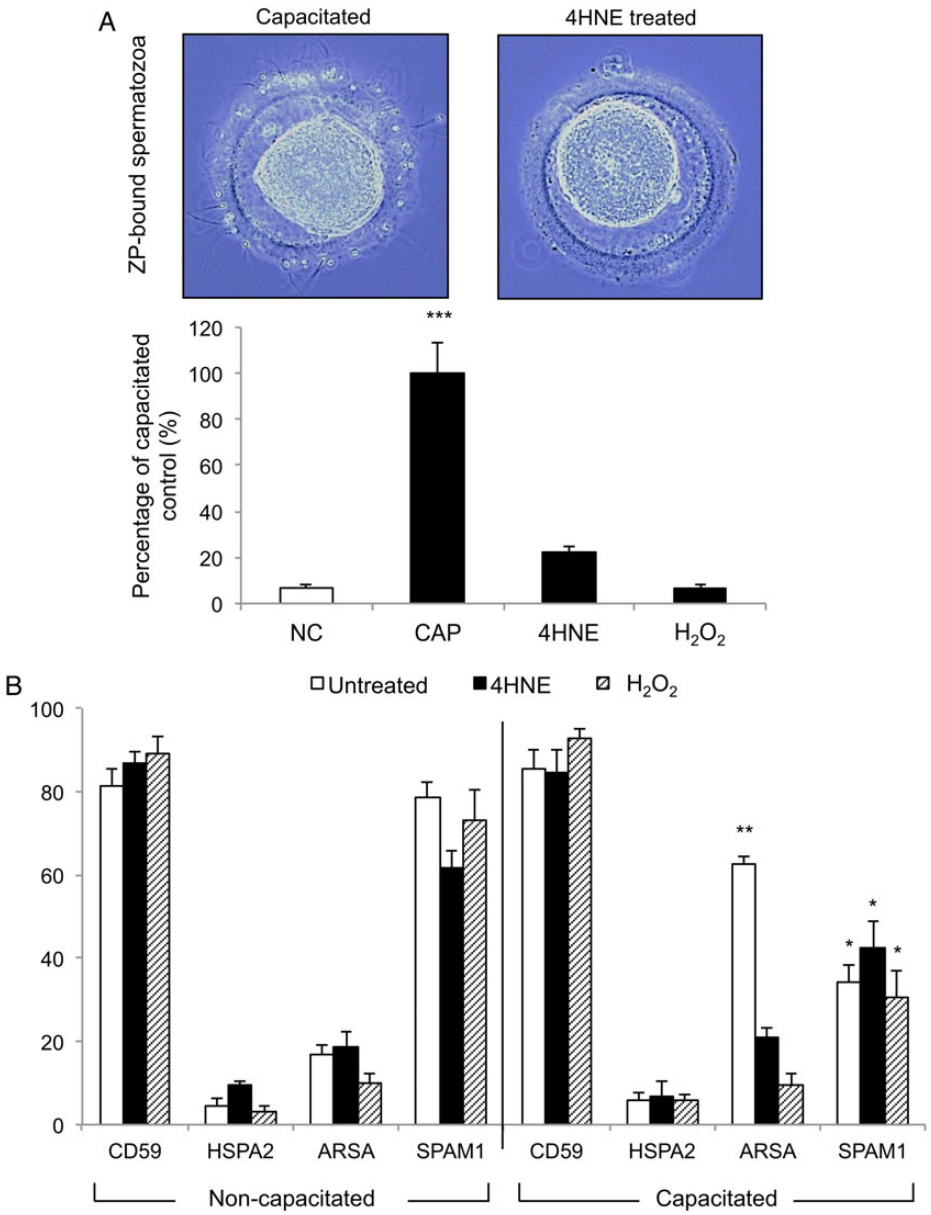

Figure 5 Assessment of zona pellucida binding competence of human spermatozoa after treatment with 4HNE and H_2O_2. Non-capacitated sperm were treated with either 50 μM 4HNE or 50 μM H_2O_2, capacitated and then (**A**) co-incubated with human zona-intact oocytes (images were taken on a fluorescence microscope using a ×40 objective) or (**B**) incubated in either anti-ARSA or anti-SPAM1 antibodies to examine the surface expression of SPAM1 and ARSA over a 180-min period of capacitation. The number of zona pellucida-bound sperm was recorded and presented as a percentage of the capacitated control. (**B**) Untreated, 4HNE-treated and H_2O_2-treated spermatozoa were incubated in capacitating media and aliquots were sampled and assessed at 0 (non-capacitated) and 180 min (capacitated) time points. The presence of SPAM1, ARSA and HSPA2 on the surface of live spermatozoa was assessed using appropriate primary antibodies, followed by an Alexa Fluor-conjugated secondary antibody and PI as a counterstain to assess cell viability. Positive control incubations were labelled with anti-CD59. The percentage of live sperm expressing surface fluorescence in each population was evaluated using a fluorescence microscope, scoring 200 cells across three biological replicates. Statistical analyses were performed using a Student's *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

treatments indicating that the induction of oxidative stress had a non-specific effect on the integrity of the plasma membrane and its capacity to express surface markers. Similarly, HSPA2 was found on the surface of <7% of spermatozoa regardless of treatment. While SPAM1 surface expression was significantly reduced in CAP cells (as shown by Redgrove *et al.*, 2012), its surface expression did not appear affected by 4HNE and H_2O_2. To ensure that the reduction in ARSA surface expression observed in response to oxidative stress did not reflect a complete loss of this protein from the cell, an aliquot of spermatozoa from each treatment was permeabilized prior to ARSA labelling. This permeabilization step resulted in distinct ARSA fluorescence over the sperm head in >85% of cells, regardless of treatment (Supplementary data, Fig. S2C).

Evaluation of protein complex assembly/function after oxidative stress

To further explore the relationship between oxidative stress and the assembly of the SPAM1/ARSA zona pellucida-receptor complex, BN-PAGE was performed with populations of spermatozoa treated with 4HNE or H_2O_2. Immunoblot experiments with anti-HSPA2 antibodies revealed that these agents did not greatly affect the profile of HSPA2-laden complexes present in spermatozoa (Fig. 6A and B) and the ~200 kDa complex containing SPAM1, ARSA and HSPA2 (Redgrove *et al.*, 2013) resolved at the correct molecular weight in all samples. Additionally, probing these BN-PAGE blots with anti-ARSA antibodies confirmed that ARSA was not lost from the complex through treatment with 4HNE and H_2O_2 (Supplementary data, Fig. S2A). These data suggest that 4HNE or H_2O_2 does not perturb the interactions that underpin the formation of sperm protein complexes. However, to determine if subtle changes in protein interactions occur within this complex after a period of oxidative stress, a proximity ligation technique was employed.

Previous studies of this complex using both proximity ligation and co-localization have revealed the close apposition SPAM1 and ARSA as well as the regulatory chaperone HSPA2 in the anterior region

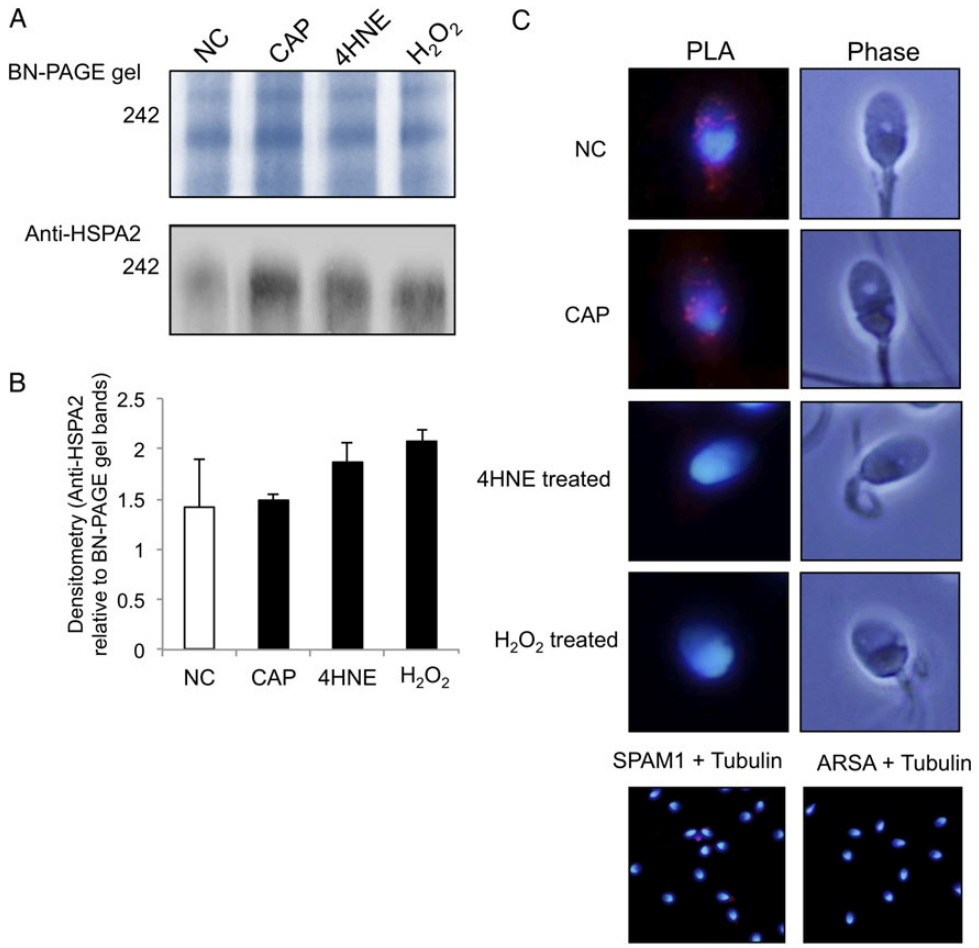

Figure 6 Effect of oxidative stress on the interaction between SPAM1 and ARSA in human spermatozoa. (**A**) Detection of 200 kDa HSPA2/SPAM1/ARSA complex in native lysates of human spermatozoa after treatment with 4HNE and H_2O_2. Aliquots of non-capacitated and capacitated spermatozoa, and capacitated spermatozoa pretreated with 4HNE or H_2O_2 were subjected to native protein lysis and the resulting protein complexes were visualized by Coomassie staining or prepared for western blotting with anti-HSPA2. Coomassie staining revealed the presence of a 200 kDa protein complex previously described by Redgrove *et al.* (2012) in native lysates of both treated and untreated samples. HSPA2 was shown to resolve in this 200 kDa complex through probing of corresponding western blots with anti-HSPA2 antibodies. Importantly, the presence of HSPA2 within this complex did not appear affected by treatment with 4HNE or H_2O_2. (**B**) This was verified through band densitometry analysis comparing the density of anti-HSPA2 labelled bands across three replicate blots to the band density of the blue native PAGE 200 kDa gel bands. (**C**) Populations of non-capacitated, capacitated, 4HNE-treated and H_2O_2-treated human spermatozoa were fixed in paraformaldehyde and allowed to settle onto poly-L-lysine-coated slides. These samples were then blocked in Duolink blocking solution, followed by incubation with primary antibodies (anti-SPAM1 and anti-ARSA; anti-SPAM1 and anti-tubulin; anti-ARSA and anti-tubulin) and oligonucleotide-conjugated secondary antibodies [proximity ligation assay (PLA) probes]. The PLA probes were then ligated and the signal was amplified according to the manufacturer's instructions (OLINK Biosciences). The fluorescent signal generated when molecules are in close association (<40 nm) was visualized using fluorescence microscopy, using either a ×100 or ×20 objective.

of the sperm head after *in vitro* capacitation (Redgrove *et al.*, 2013). To further evaluate the effect of oxidative stress on interactions between ARSA and SPAM1, the Duolink PLA was performed on non-capacitated, capacitated, 4HNE-treated and H_2O_2-treated cells. As shown in Fig. 6C, the punctate fluorescent spots that indicate a close interaction between two proteins (<40 nm apart) were no longer detectable over the anterior head region of spermatozoa treated with either 4HNE or H_2O_2. This suggests that SPAM1 and ARSA were not able to form a stable interaction in a majority of 4HNE- or H_2O_2-treated human spermatozoa, with only 8 and 12% of cells showing evidence of PLA fluorescence, respectively (data not shown). This was in contrast to both NC and CAP sperm where 65 and 76% of cells, respectively, showed evidence of an interaction between SPAM1 and ARSA, as indicated by the red fluorescence within the sperm head (Fig. 6C). Importantly, the specificity of this reaction was confirmed through dual labelling with a combination of the anti-SPAM1 and anti-ARSA antibodies with that of an unrelated antibody (anti-tubulin). These results suggested that rather than a complete dysregulation of this zona pellucida complex assembly, the reduction of ARSA expression on the surface of capacitated human spermatozoa may be due to a loss of the tight coordination/association between members of this complex during capacitation in cells experiencing the effects of oxidative stress.

Investigation of HSPA2 as a target for modification by 4HNE after exposure to oxidative stress

As our model suggests that the chaperone activity of HSPA2 is required for the co-ordinated presentation of ARSA on the sperm surface, we followed up this series of experiments investigating potential impacts of oxidative stress on HSPA2.

In order to investigate the potential adduction of HSPA2 by 4HNE in capacitated and oxidative stressed human spermatozoa, an immunoprecipitation strategy was adopted. Human sperm lysates extracted from both H_2O_2-treated and capacitated cells were immunoprecipitated with anti-HSPA2 antibodies and eluted proteins were sequentially probed with anti-HSPA2 (to confirm the specificity of the IP; Fig. 7A) and anti-4HNE (Fig. 7B) to identify potential protein adducts. As shown in Fig 7A and B, the HSPA2 protein was effectively isolated as a predominant band at ~70 kDa in the eluates from both CAP- and H_2O_2-treated cells. An additional band of ~55 kDa was also detected in these samples; however, this may correspond to the heavy IgG chain given that a band of similar size was detected in the antibody-only control. The specificity of this immunoprecipitation was confirmed through the use of antibody-only and bead-only controls, as well as a 'precleared' control, each of which failed to show any labelling of the 70 kDa band corresponding to HSPA2. Interestingly, upon probing a corresponding blot with anti-4HNE, bands of the appropriate molecular weight for HSPA2 were also detected in both the CAP- and H_2O_2-treated sperm eluates. Additionally, these bands were clearly more heavily labelled in the eluate from human spermatozoa treated with H_2O_2 prior to capacitation. Importantly, equal loading of eluted proteins was confirmed through silver staining of the corresponding gel. This result identifies HSPA2 as a key target for modification by the aldehyde 4HNE in human spermatozoa subjected to oxidative stress. To confirm that 4HNE was not directly targeting ARSA and resulting in an inability of this protein to be expressed on the cell surface, proteins immunoprecipitated with anti-4HNE were also probed for the presence of ARSA. While bands were detected in the elution lanes at the appropriate size (~55 kDa), ARSA appeared to be constitutively modified by 4HNE with no change detected between CAP sperm lysates and lysates from H_2O_2-treated spermatozoa (Supplementary data, Fig. S2B).

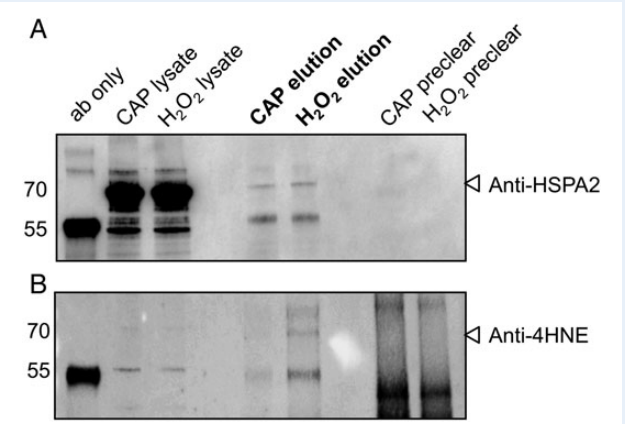

Figure 7 Examination of HSPA2/4HNE interaction in human spermatozoa. Lysates of capacitated and H_2O_2-treated, capacitated human spermatozoa were incubated with protein G Dynabeads conjugated with anti-HSPA2 antibodies. The beads were washed, and then bound proteins were eluted and resolved on SDS–PAGE gels before being transferred to nitrocellulose membranes. (**A**) Membranes were probed with anti-HSPA2 antibodies to confirm the efficacy of immunoprecipitation before being stripped and then reprobed with 4HNE antibodies (**B**). Controls included an antibody-only control (Ab only) in which antibody-conjugated beads were incubated in the absence of cell lysate. In addition, a whole sperm lysate was included to confirm the identity of the co-precipitated proteins as well as a preclear eluate control. The experiment was replicated three times using pooled semen samples and representative blots are depicted.

Given these results it would be tempting to speculate that 4HNE adduction to the HSPA2 chaperone may give rise to the loss of sperm–zona pellucida recognition observed in this study. If this was the case, we reasoned that it would be possible to prevent the effects of this lipid aldehyde by introducing nucleophilic scavengers to limit its bioavailability. Penicillamine has previously been shown to significantly reduce the cellular expression of 4HNE owing to its ability to covalently bind the aldehyde (Aitken *et al.*, 2012a). In view of this protective effect, the remaining experiments of this study focused on analysing whether penicillamine could ameliorate the negative outcomes of 4HNE adduction to HSPA2, ARSA surface expression and zona pellucida binding in oxidatively stressed human spermatozoa.

The effect of penicillamine treatment on zona pellucida binding, ARSA surface expression and 4HNE adduction of HSPA2

To evaluate the effect of penicillamine on ARSA expression on the sperm surface, live human spermatozoa were labelled with anti-ARSA and examined through fluorescence microscopy. These analyses revealed a

significant increase in the number of labelled spermatozoa after addition of both 0.5 and 1 mM penicillamine when compared with H_2O_2-treated sperm. Indeed, incubation with 0.5 and 1 mM penicillamine resulted in a 2- and 3-fold increase in the number of spermatozoa surface labelled with ARSA compared with that of the H_2O_2-treated sperm population ($P = 0.05$; $P = 0.01$), respectively (Fig. 8A).

To assess whether these effects were related to a relief of 4HNE adduction to HSPA2, immunoprecipitation was performed using

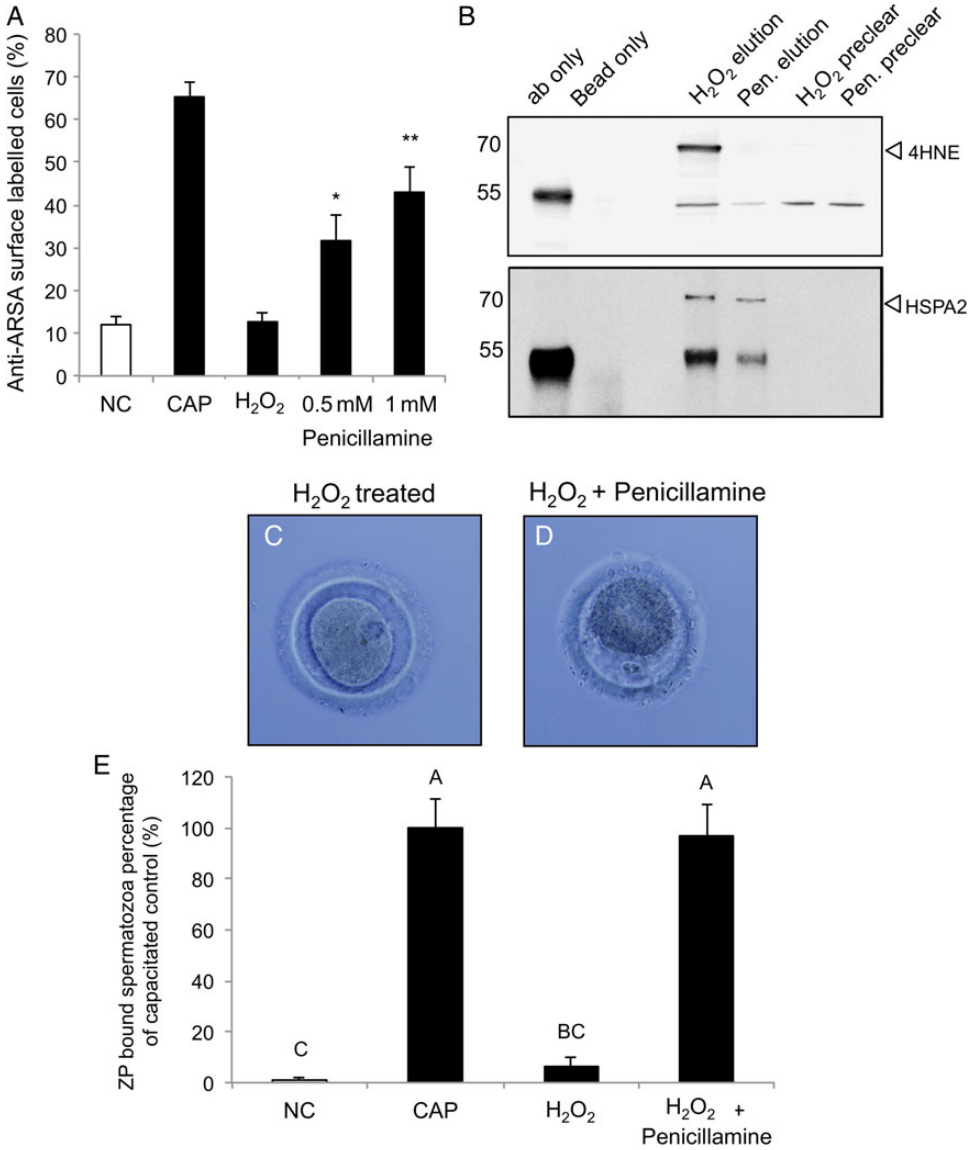

Figure 8 Treatment with penicillamine reverses the effects of H_2O_2 induced 4HNE adduction and recovers the zona pellucida binding ability of oxidative stressed spermatozoa. Following the induction of oxidative stress with H_2O_2 treatment, cells were washed once in BWW and then resuspended in capacitating medium supplemented with penicillamine. H_2O_2-treated spermatozoa were in contact with penicillamine for the duration of this 3 h incubation after which they were washed once in BWW in preparation for further analyses. (**A**) Untreated (CAP), H_2O_2-treated (H_2O_2) and H_2O_2/penicillamine (0.5 or 1 mM)-treated spermatozoa were incubated in capacitating media and aliquots were sampled and assessed after 180 min. NC spermatozoa were incubated under non-capacitating conditions for the same duration. The presence of ARSA on the surface of live spermatozoa was assessed by labelling with anti-ARSA primary antibodies, followed by a FITC-conjugated secondary antibody and PI as a counterstain to assess cell viability. The percentage of live sperm expressing surface fluorescence in each population was evaluated using a fluorescence microscope, scoring 200 cells across three biological replicates. *$P < 0.05$; **$P < 0.01$. (**B**) H_2O_2-treated and H_2O_2/penicillamine-treated spermatozoa were lysed and immunoprecipitated with anti-HSPA2 antibody. Eluates were probed with anti-4HNE and then stripped and re-probed with anti-HSPA2 to confirm the efficacy of the IP. Ab-only, bead-only and precleared controls were resolved alongside these eluted proteins to confirm IP specificity. (**C**–**E**) Spermatozoa from each treatment and a NC control were co-incubated with homologous human zona pellucida-intact oocytes and the number of motile spermatozoa bound to each egg were recorded across each replicate and plotted as a percentage of the capacitated control. (A–C) Groups without a common subscript differ significantly, $P < 0.01$. Images were taken on a fluorescence microscope using a ×40 objective.

anti-HSPA2. Through immunoblotting analysis with anti-HSPA2, 70 kDa bands were observed in both H_2O_2-treated and H_2O_2/penicillamine-treated eluates (Fig. 8B). However, when these eluted proteins were probed with anti-4HNE antibodies, a distinct loss of 4HNE adducts was revealed in the penicillamine-treated sperm eluate.

Given these results, we sought to test the ability of penicillamine to limit the impact of H_2O_2 on sperm function by examining the ability of these cells to interact with homologous zonae pellucidae. Surprisingly, while H_2O_2-treated cells showed a marked reduction in zona pellucida binding ability compared with the CAP sperm control (6% of CAP control; Fig. 8C and E), the co-incubation of capacitating sperm with penicillamine enabled a near complete recovery of zona pellucida binding ability (97% of capacitated control; Fig. 8D and E). This result was statistically significant from both the non-capacitated control and H_2O_2-treated cells, while the capacitated control and penicillamine-treated cells were statistically similar. Importantly, these results were not mediated by differences in sperm total motility or hyperactivated motility, as shown in Supplementary data, Table SI.

Discussion

ROS are well recognized as key regulators of mammalian sperm capacitation, yet a fine balance exists between a 'beneficial' presence of ROS to promote cholesterol efflux (Boerke *et al.*, 2013), cAMP production (Zhang and Zheng, 1996; Ickowicz *et al.*, 2012), protein tyrosine phosphorylation (Aitken *et al.*, 1998; Leclerc *et al.*, 1997) and the acrosome reaction (de Lamirande *et al.*, 1998) and the 'detrimental' presence of excess ROS that can lead otherwise viable cells down an intrinsic apoptotic-like pathway (Aitken *et al.*, 2012b). The present study suggests that levels of oxidative stress that might be encountered *in vivo* (Uchida, 2003; Aitken *et al.*, 2012b) have relatively little effect on events associated with early capacitation, such as membrane fluidity, raft redistribution and protein tyrosine phosphorylation. However, such low levels of stress can, nevertheless, have detrimental downstream effects on sperm–zona pellucida interaction. Moreover, this study has suggested that the inhibition of zona pellucida binding under these circumstances is associated with the impaired expression of the zona pellucida receptor, ARSA, on the sperm surface.

The disrupted presentation of ARSA on the sperm surface is, in turn, thought to be associated with the dysregulation of a sperm–zona pellucida-receptor complex that becomes expressed during capacitation comprising the regulatory chaperone HSPA2 in close association with SPAM1/ARSA (Redgrove *et al.*, 2012, 2013). Following exposure to oxidative stress, this complex does not disappear; however, its molecular constituents no longer exist in sufficient proximity to one another to give a positive signal when interrogated in a PLA. This loss of functional association is in turn thought to be due to the post-translational modification of HSPA2 as a consequence of adduction by the lipid aldehyde, 4HNE.

4HNE is the major cytotoxic aldehyde generated by the attack of free radicals on ω-6 polyunsaturated fatty acids during lipid peroxidation and accumulates more readily in biomembranes than in free solution due to its lipophilic nature (Esterbauer et al., 1982; Esterbauer et al., 1986; Uchida, 2003). The molecule itself exhibits a wide range of biological functions such as the inhibition of protein and DNA synthesis, inactivation of enzymes and stimulation of phospholipase C (Uchida, 2003; Carini *et al.*, 2004). However, in addition to these functions, the electrophilic nature of 4HNE causes it to form stable covalent adducts with the nucleophilic functional groups of cysteine, lysine and histidine residues of numerous proteins to form both Michael and Schiff base adducts, thereby introducing carbonyl groups into proteins and altering their function (Uchida and Stadtman, 1992; Butterfield, 2002; Perluigi *et al.*, 2012).

Although there are several potential explanations for the loss of ARSA surface expression following oxidative stress, the increase in HSPA2-bound 4HNE adducts supports a mechanism involving non-enzymatic post-translational protein modification by 4HNE. Certainly, the use of the antioxidant penicillamine to counteract the deleterious effects of H_2O_2 on sperm function suggests that this damage is mediated by electrophilic aldehyde(s). Penicillamine has been shown to significantly reduce the expression of 4HNE in human spermatozoa by intercepting 4HNE as it is produced, through covalent interaction, while simultaneously chelating transition metals, such as iron and copper, that catalyse the cascades of lipid peroxidation that are responsible for the formation of these aldehydes (Aitken *et al.*, 2012a). Our immunoprecipitation analyses suggest that the use of penicillamine during human sperm capacitation was able to protect/relieve HSPA2 from 4HNE adduction and subsequently a majority of H_2O_2-treated cells were able to present ARSA on their surface and participate in zona pellucida recognition. In light of this, we propose that oxidative stress induces lipid peroxidation and the subsequent generation of 4HNE (and likely other reactive aldehydes) targets HSPA2, causing modifications to the protein and consequently its molecular chaperone activity. Such modifications then appear to destroy the ability of this chaperone to orchestrate the reciprocal surface expression of ARSA and SPAM1 (Redgrove *et al.*, 2012, 2013).

Although investigating potential sites of 4HNE adduction and the manner in which these modifications may compromise the structure and/or function of HSPA2 is beyond the scope of this study, in a number of independent studies 4HNE has been shown to interfere with the activities of various signalling kinases, such as mitogen-activated protein kinases (MAPKs; Sampey *et al.*, 2007) and protein kinase C (Harry *et al.*, 2012), and to regulate or dysregulate cell processes such as apoptosis, proliferation and differentiation (Leonarduzzi *et al.*, 2004). It is likely that 4HNE adduction could modify the ATPase activity of HSPA2 and thus prevent it from fulfilling its role in protein trafficking or refolding events, as has been previously documented for HSP72 in the rat (Carbone *et al.*, 2004, 2005). In these studies, 4HNE modification of cytosolic HSP72 led to an inhibition of protein refolding function through covalent modification of Cys267 in the ATP binding site of the molecule, purportedly through a thiol-specific mechanism of inactivation (Carbone *et al.*, 2004). Moreover, in a rat model of alcohol-induced oxidative stress (Carbone *et al.*, 2005) it has been demonstrated that 4HNE deregulates the activity of an alternative heat shock protein, HSP90, through a similar mode of thiol modification.

Taken together, these data suggest that heat shock proteins are key targets for 4HNE adduction reactions and that their chaperoning activity is particularly susceptible to such an insult. While similar studies need to be carried out with human HSPA2 to determine whether equivalent mechanisms may contribute to its dysregulation, it is tempting to conclude that 4HNE adduction directly leads to a loss of HSPA2 chaperone activity and a subsequent perturbation of zona pellucida protein complex assembly.

Within the patient population levels of oxidative stress are, of course, variable (Aitken et al., 2010) and the suite of proteins that are usually targeted for adduction by cytotoxic aldehydes will vary as a consequence. Although this study did not seek to identify additional proteins or protein complexes that interact with 4HNE, this may be a useful approach to determine whether proteins involved in the initiation of sperm plasma membrane remodelling events may also be compromised by reactive aldehyde modification in the same manner as HSPA2. As 4HNE has been implicated in the inactivation of several key kinases, the modification of PKA or PKC activity by reactive aldehydes may also have downstream effects. Specifically, this may affect the phosphorylation status of key proteins involved in the activation of chaperone proteins, such as HSPA2, responsible for the remarkable membrane modification and protein reshuffling events that are crucial for the success of sperm–zona pellucida interaction (Lefievre et al., 2004).

In conclusion, this series of experiments has established a clear link between oxidative stress, impaired function of the SPAM1/ARSA/HSPA2 zona pellucida-receptor complex in human spermatozoa and a severely reduced ability to recognize and adhere to the zona pellucida. This suggests that the coordinated functions of both SPAM1 and ARSA

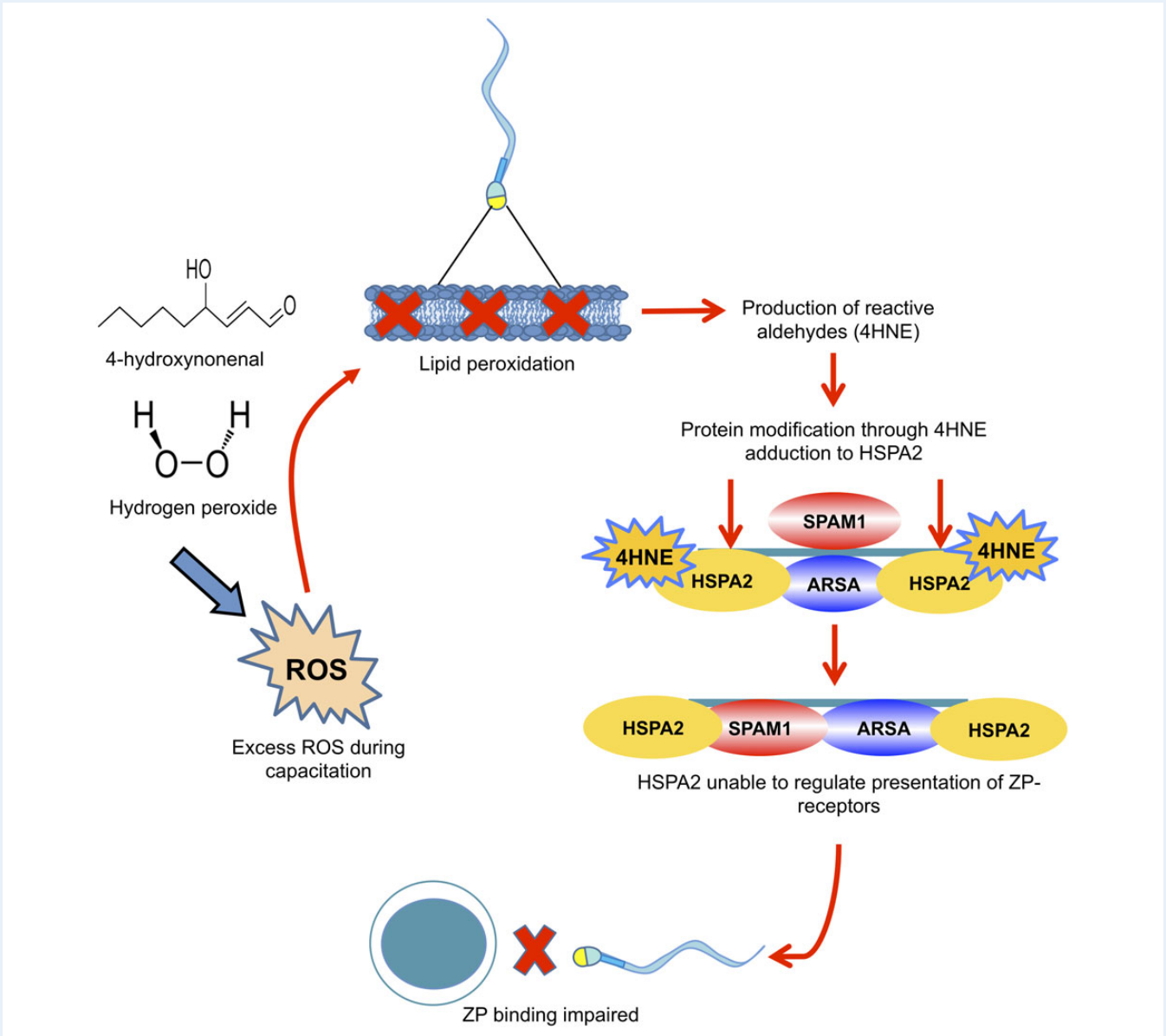

Figure 9 Model of impaired zona pellucida interaction due to oxidative stress during sperm capacitation. Incubation of sperm in the presence of the reactive aldehyde 4HNE or H_2O_2 results in the production of mitochondrial and cytoplasmic ROS. This often leads to a loss of mitochondrial membrane potential, a loss of motility, lipid peroxidation and the production of further reactive aldehydes such as 4HNE. While this commonly leads to DNA damage, it can also lead to the modification of proteins through protein adduction that can alter their functions. The heat shock protein 70 family of chaperones are key targets for 4HNE adduction (Aitken et al., 2012a) and hence we propose in this model that adduction of HSPA2 by 4HNE may result in a loss of chaperone activity and a consequential inability to coordinate the presentation of zona pellucida receptors on the sperm surface in preparation for sperm–zona pellucida adhesion.

may play a vital role in the initial tethering of human spermatozoa to the zona pellucida. Moreover, we propose that the dysregulation of SPAM1 and ARSA complex assembly and/or presentation may be linked to a reduction in the chaperone function of HSPA2 owing to its modification by the cytotoxic lipid peroxidation product 4HNE (Fig. 9). Given that diminished HSPA2 expression in the sperm of infertile men has been causally linked to a loss of zona pellucida binding ability (Huszar *et al.*, 2006; Redgrove *et al.*, 2012), the implications of this work may extend to an increase in understanding of male factor infertility, particularly in patients that present with this particular defect. Certainly, this study suggests that the chaperone HSPA2 is sensitive to the effects of oxidative stress, highlighting an important direction for further study into a causative link between non-enzymatic post-translational modifications and a loss of the functional presence of HSPA2 in cases of male infertility.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

E.G.B. contributed to study design, conducted the experiments and generated the manuscript. R.J.A. contributed to study conception and design, data interpretation and manuscript editing. A.L.A. provided technical assistance. E.A.M. contributed to study design and data interpretation and B.N. contributed to study design, data interpretation, manuscript preparation and editing.

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Conflict of interest

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

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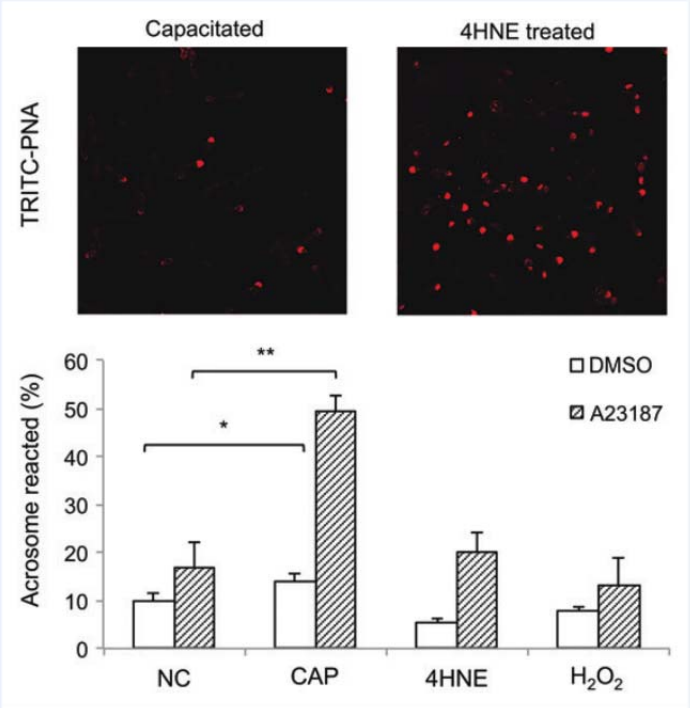
Chapter 2: Supplementary material



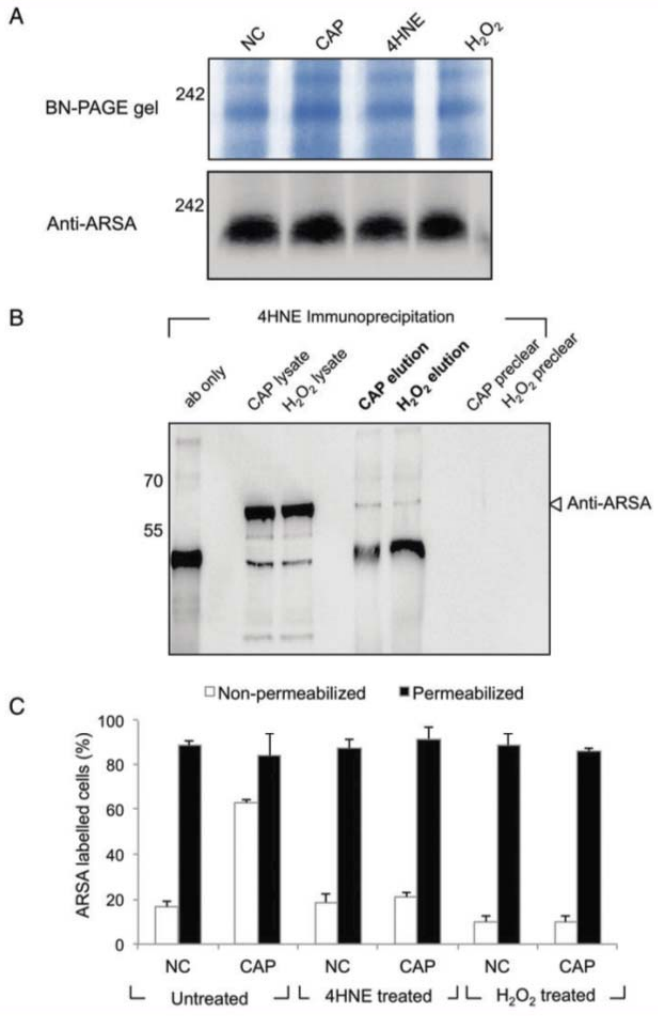
Supplementary Figure S1 Assessment of acrosome reaction competence of human spermatozoa after treatment with 4HNE and H_2O_2. Non-capacitated spermatozoa were treated with either 50 μM 4HNE or 50 μM H_2O_2, capacitated and then treated with either calcium ionophore (A23187) to induce acrosomal exocytosis or a DMSO vehicle control. Acrosome-reacted cells were recorded through the use of TRITC-labelled Peanut Lectin Agglutinin (PNA). An absence of PNA labelling over the acrosomal region indicated an acrosome-reacted cell. Statistical analyses were performed using a Student's *t*-test, $^*P < 0.05$, $^{**}P < 0.01$.

Supplementary Table S1 Motility of human spermatozoa after capacitation.

	Total motile (%)	VCL (μm/s)	ALH (μm)	Hyperactivated (%)
NC	82	54.8	3.5	8
CAP	75	181.2	8.1	36
4HNE	72	169.5	6.9	32
H_2O_2	73	183.2	7.2	38
1 mM Pen.	71	196.8	7.6	34

Immediately prior to zona pellucida binding assessments of human spermatozoa, sperm were assessed for total motility (%), velocity (VCL; μm/s) and amplitude of lateral head displacement (ALH; μm) using an HTM-IVOS CASA system. The percentage of cells displaying hyperactivated movement was also assessed using phase contrast microscopy and recorded for 100 cells.
NC, non-capacitated; CAP, capacitated; 4HNE, 4-hydroxynonenal; Pen, penicillamine.

Chapter 2: Supplementary material



Supplementary Figure S2 Examination of the effect of oxidative stress on ARSA. (**A**) Detection of ARSA in a 200 kDa complex in native lysates of human spermatozoa after treatment with 4HNE and H_2O_2. Populations of non-capacitated, capacitated, 4HNE treated and H_2O_2 treated spermatozoa were subjected to native protein lysis and the resulting protein complexes were resolved by BN-PAGE before being visualized by Coomassie staining or prepared for western blotting with anti-ARSA antibodies. Coomassie staining revealed the presence of a 200 kDa protein complex previously described by Redgrove *et al.* (2012) in native lysates of both treated and untreated samples. ARSA was shown to resolve in this 200 kDa complex through probing of corresponding western blots with anti-ARSA antibodies. Importantly, the expression of ARSA within this complex was not influenced by treatment with 4HNE or H_2O_2. (**B**) Lysates of capacitated and H_2O_2 treated, capacitated human spermatozoa were incubated with protein G Dynabeads conjugated with anti-4HNE antibodies. The beads were washed, and then bound proteins were eluted and resolved on SDS–PAGE gels before being transferred to nitrocellulose membranes. Membranes were probed with anti-ARSA antibodies to investigate an interaction between 4HNE and ARSA as a putative explanation for the loss of ARSA surface expression observed in 4HNE and H_2O_2 treated cells. Results revealed the presence of ARSA in the 4HNE immunoprecipitated protein elution lanes. However, the presence of ARSA was not affected by H_2O_2 treatment suggesting that ARSA may be constitutively modified by 4HNE in human spermatozoa. Negative controls included an antibody only control (Ab only) in which antibody-conjugated beads were incubated in the absence of cell lysate and a bead only control (bead only) in which non-conjugated beads were incubated with sperm lysate. A whole sperm lysate was included to confirm the identity of the co-precipitated proteins as was the washed material recovered after resuspension of the beads in lysis buffer to confirm the specificity of the elution as well as a preclear eluate control. (**C**) To ensure that the reduction in ARSA surface expression observed in response to oxidative stress did not reflect a complete loss of this protein from the cell, an aliquot of spermatozoa from each treatment was permeabilized with 0.02% Triton X-100 prior to incubation with anti-ARSA antibodies. This permeabilization step resulted in distinct ARSA fluorescence over the sperm head in >85% of cells, regardless of treatment with 4HNE or H_2O_2.

CHAPTER 3:

A novel characterization of the HSPA2-stabilizing protein BAG6 in human spermatozoa

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Chapter 3: Overview

In the studies described in chapter two we established that the chaperone HSPA2 is highly sensitive to damage by the lipid peroxidation product 4HNE. Importantly, the stress-driven interaction of these two molecules may result in the attenuation of HSPA2-mediated sperm surface remodeling events that lead to a severe loss in sperm-egg recognition in human spermatozoa. From these studies, we became interested in evaluating oxidative stress mediated pathways by which the protein expression of HSPA2 may be disrupted in cases of male infertility. In cells that possess an innate proteasome, the modification of vulnerable proteins by 4HNE has been demonstrated to induce protein degradation. Interestingly, in the mouse testis, the stability of HSPA2 is tightly regulated by the actions of an additional chaperone BCL-2 associated athanogene 6 (BAG6) with the absence of BAG6 expression in germ cells resulting in the rapid ubiquitination and degradation of HSPA2 by the proteasome. In this context, the aim of this manuscript was to determine the presence of BAG6 in the human male germ-line and explore a relationship between BAG6 and HSPA2 during sperm development.

The results of this study describe a stable interaction between HSPA2 and BAG6 in human testicular germ cells and epididymal spermatozoa that is sustained in mature sperm throughout capacitation. This is highly suggestive of a conserved role for BAG6 in the maintenance of HSPA2 stability in human germ cells. Additionally, during sperm capacitation, a change in BAG6 localization was revealed such that an additional pool of immunoreactive protein was detected in the apical sperm head. This relocation or unmasking event corresponds temporally with the activation of HSPA2 and leads us to postulate that the presence of BAG6 is also important in the prevention of protein misfolding or mislocalization events during the assembly of HSPA2-laden zona pellucida-receptor complexes. In this way the presence of BAG6 may be critical for not only the stability of HSPA2 during testicular sperm maturation but also to maintain the function of this chaperone during sperm surface remodeling prior to zona pellucida interaction.

The particular relevance of this study to human fertility was realized through examination of BAG6 in the spermatozoa of infertile men. This revealed that spermatozoa that lack the ability to bind to the zona pellucida, through a lack of HSPA2 expression, also had a severe deficiency in the expression of BAG6. These data lead us to propose that BAG6 may be a new target for idiopathic male infertility and that the absence of BAG6 could lead to the accelerated degradation of HSPA2 in human germ cells

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ORIGINAL RESEARCH

Novel characterization of the HSPA2-stabilizing protein BAG6 in human spermatozoa

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ABSTRACT: While a large cohort of sperm surface receptors underpin sperm–oocyte adhesion processes, our recent work has revealed that the molecular chaperone Heat Shock Protein A2 (HSPA2) is a key regulator of zona pellucida-receptor complex assembly in our own species. Indeed, in the infertile population, spermatozoa that fail to interact with the zona pellucida of the oocyte consistently lack HSPA2 protein expression. While the mechanisms behind this protein deficiency are under consideration, BCL2-associated athanogene 6 (BAG6) has been identified as a key regulator of HSPA2 stability in mouse germ cells. However, in the human, the presence of BAG family proteins remains completely uncharacterized. Consequently, this study aimed to determine the presence of BAG6 in human sperm cells and to characterize its putative interaction with HSPA2 throughout sperm cell development. BAG6 was shown to co-localize with HSPA2 in human testicular germ cells and epididymal spermatozoa. Similarly, BAG6 was identified in the equatorial region of non-capacitated spermatozoa but underwent a marked relocation to the anterior region of the head upon the induction of capacitation in these cells. Protein–protein interaction assays revealed the stable interaction of BAG6 and HSPA2 proteins in mature spermatozoa. Furthermore, examination of the spermatozoa of infertile men with zona pellucida binding defects, related to a lack of HSPA2, revealed a concomitant deficiency in BAG6 protein expression. In view of the findings described in this study, we propose that BAG6 is likely a key regulator of HSPA2 stability/function in human germ cells. Moreover, its under-representation in spermatozoa with zona pellucida binding deficiency suggests that BAG6 may be an important candidate to study for a further understanding of male idiopathic infertility.

Key words: BAG6 / HSPA2 / sperm–oocyte interaction / male infertility

Introduction

The ability of spermatozoa to recognize and bind the zona pellucida of an oocyte represents an important physiological barrier to fertilization. Indeed, in species such as the human and mouse, it has been shown that spermatozoa that are capable of binding to the zona pellucida have a significantly higher degree of DNA integrity than those gametes that are unable to interact with homologous oocytes (Liu and Baker, 2007; Kumar *et al.*, 2013). Such findings are supported by large-scale epidemiological studies that have shown an elevated risk of birth defects in children conceived by techniques such as intracytoplasmic sperm injection that bypass this natural barrier to fertilization (Davies *et al.*, 2012). It is therefore concerning that one of the most common lesions identified in the sperm of men attending IVF clinics is an idiopathic failure of sperm–oocyte recognition (Liu and Baker, 2000). Although this complex lesion remains relatively unexplored, concomitant with a loss of zona pellucida recognition is a striking under-representation of the important regulatory chaperone Heat Shock Protein A2 (HSPA2) in the spermatozoa of these patients (Redgrove *et al.*, 2012). Moreover, the expression of this chaperone has been shown to be highly predictive of sperm maturity and IVF success (Huszar *et al.*, 2000; Ergur *et al.*, 2002; Cayli *et al.*, 2003).

For these reasons, significant consideration has been given to the functional role of HSPA2 both in sperm development (Huszar *et al.*, 1997, 2000, 2006; Tian *et al.*, 2014; reviewed by Scieglinska and Krawczyk, 2015) and in sperm–zona pellucida interaction (Redgrove *et al.*, 2013). HSPA2 is a testis-enriched member of the HSP70 family of chaperones, which is primarily responsible for preventing the aggregation of misfolded proteins (Mayer and Bukau, 2005) and for fulfilling secondary roles in the transmembrane transport of client proteins (Huszar *et al.*, 2000; Dun *et al.*, 2012) and the formation of multimeric protein complexes (Redgrove *et al.*, 2011). The importance of HSPA2 for sperm development has been highlighted by targeted ablation of the gene, which results in a phenotype of complete male infertility (Dix *et al.*, 1997). A conserved role in our own species is suggested by the low levels of *Hspa2* gene expression that have been documented in individuals

suffering from conditions such as oligozoospermia and complete azoospermia (Cedenho et al., 2006).

In post-meiotic germ cells, aberrant HSPA2 expression has been linked to defects in spermiogenesis (Huszar et al., 1997), a process that results in DNA condensation and plasma membrane remodeling to form specialized zona pellucida and hyaluronic acid binding domains on the sperm head (Huszar et al., 2000, 2007). During this phase of sperm development, HSPA2 is responsible for chaperoning DNA repair enzymes and structural proteins required for sperm remodeling as well as spermatid-specific DNA packaging transition proteins. Consequently, spermatozoa lacking HSPA2 commonly possess heavily fragmented DNA and oxidative DNA lesions (Cayli et al., 2004). Similarly, these cells also present with reduced capacity to bind to hyaluronic acid polymers and zonae pellucidae (Huszar et al., 2006). Interestingly, these latter functions have also been causally related to a novel, extra-testicular role for the HSPA2 protein in mature spermatozoa, whereby the chaperone assists in the assembly and presentation of zona-recognition complexes at the sperm surface (Redgrove et al., 2012, 2013).

Hence, a major goal of ongoing investigations is to determine how the incorporation of HSPA2 into the differentiating gamete becomes so dramatically disrupted in cases of infertility. The putative mechanisms that compromise HSPA2 expression in human spermatozoa include genetic/epigenetic mutations and damage to the mRNA transcripts and/or the machinery involved in their translation. Alternatively, the loss of HSPA2 may be attributed to the targeted destruction of the protein itself. The latter possibility is supported by studies of germ cell development, which have shown the stability of the HSPA2 protein is reliant upon its interaction with the co-chaperone BAG6 (BCL2-associated athanogene 6; formerly BAT3, HLA-B-associated transcript 3) (Sasaki et al., 2008; Kawahara et al., 2013). Importantly, targeted deletion of *Bag6* results in the rapid polyubiquitination and subsequent degradation of HSPA2 (Sasaki et al., 2008), leading to an infertility phenotype that mirrors that of *Hspa2* null males. These findings raise the possibility that BAG6 may be a molecular target for idiopathic male infertility (Sasaki et al., 2008). However, the presence of BAG family proteins remains completely uncharacterized in human germ cells.

In this study, we aimed to determine the presence of BAG6 in the human testis and to investigate its putative interaction with HSPA2 during the development of human spermatozoa. Our results demonstrate that BAG6 is present in human testicular germ cells, and in the sperm cells present in the epididymis and collected after ejaculation. The BAG6 and HSPA2 proteins were also co-localized in testicular germ cells and were shown to form a stable interaction that persisted in ejaculated spermatozoa. These results support a conserved role for BAG6 in the regulation of HSPA2 protein stability in the human and mouse testis and raise the intriguing prospect that BAG6 function may extend to the modulation of HSPA2 chaperoning activity in mature human spermatozoa.

Materials and Methods

Ethics

The experiments described in this study were performed with human semen samples obtained with informed written consent from either a panel of healthy normozoospermic donors or IVF patients enrolled in ART programs with IVFAustralia (Greenwich, NSW, Australia). Volunteer involvement and all experimental procedures were performed in strict accordance with institutional ethics approvals granted by the University of Newcastle Human Research and Ethics Committee and the IVFAustralia Ethics Committee. All sperm samples were subjected to analyses in accordance with the World Health Organisation guidelines (WHO, 2010), and patients were selected on the basis of failed IVF associated with poor zona pellucida adherence following overnight incubation with a minimum of five oocytes.

Reagents

Unless specified, chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of research grade. The following primary antibodies were purchased to characterize proteins of interest: rabbit polyclonal anti-HSPA2 (Sigma Cat # SAB1405970); mouse monoclonal antibody to BAG6 (sc-365928; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat polyclonal antibody to ADAM30 (sc-26021, Santa Cruz Biotechnology). Albumin and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Research Organics (Cleveland, OH, USA); D-glucose, sodium hydrogen carbonate, sodium chloride, potassium chloride, calcium chloride, potassium orthophosphate and magnesium sulfate were all of analytical reagent grade, purchased from Merck (BDH Merck, Kilsyth, VIC, Australia). Tris was from ICN Biochemicals (Castle Hill, NSW, Australia), and Percoll from GE Healthcare (Rydalmere, NSW, Australia). Nitrocellulose was from GE Healthcare (Buckinghamshire, UK) while highly pure Coomassie Brilliant Blue G250 was obtained from Serva (Heidelberg, Germany). Mowiol 4-88 was from Calbiochem (La Jolla, CA, USA), and paraformaldehyde was supplied by ProSciTech (Thuringowa, QLD, Australia). Protein G beads were purchased from Life Technologies (Carlsbad, CA, USA) and 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP) was purchased from Thermoscientific (Waltham, MA, USA). Appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology) and Sigma-Aldrich.

Sperm preparation and capacitation

Human spermatozoa were acquired from normozoospermic donors as part of the University of Newcastle donor program. Spermatozoa were prepared using Percoll density gradient centrifugation as described previously (Redgrove et al., 2012). Good quality spermatozoa from the 80% Percoll fraction were retrieved, washed and assessed for motility and morphology through light microscopy analyses (Olympus CX40). Cells were induced to capacitate as previously described (Redgrove et al., 2012) or held in a non-capacitated state in bicarbonate free Biggers, Whitten and Whittingham medium [Non-capacitating BWW; (Biggers et al., 1971)], composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin, 20 mM HEPES buffer and 1 mg/ml polyvinyl alcohol (osmolarity of 300 mOsm/kg). For the purpose of these experiments, each biological replicate was performed on spermatozoa pooled from two ejaculates to account for intra-donor variability and a minimum of three biological replicates were performed.

Immunohistochemistry and tissue morphology assessment

Human testis and epididymis tissue sections used throughout this study were purchased from Abcam (Ab4373; Ab4326), and the embedded tissue was dewaxed and rehydrated as previously described (Reid et al., 2012).

For immunolocalization studies, sections were subjected to antigen retrieval via immersion in 10 mM sodium citrate (pH 6) and microwaving for 10 min at 1000 W. Subsequent incubations were performed as described by Reid et al. (2012) with primary antibodies diluted 1:50 (BAG6) or 1:100

(HSPA2) overnight at 4°C. Following incubation with appropriate Alexa Fluor conjugated secondary antibodies (1:200), the slides were washed three times and incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000) for 5 min, mounted using a MOWIOL anti-fade reagent (13% Mowiol 4-88, 33% glycerol, 66 mM Tris, pH 8.5, 2.5% 1,4-diazabicyclo-[2.2.2]octane) and viewed on an Olympus FV1000 confocal microscope using a 175 60×/1.2 NA UPLSAPO oil immersion objective lens (Olympus America, Centre Valley, PA, USA).

For morphology analyses, haematoxylin and eosin staining was performed (Fischer *et al.*, 2008) and slides were mounted in DPX and dried overnight in a fume hood before being viewed with an Avio Imager A1 fluorescence microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA), and images were taken using an Olympus DP70 microscope camera (Olympus America).

Immunocytochemistry

Human spermatozoa were fixed in 4% paraformaldehyde, washed in 0.05 M glycine/PBS and allowed to settle on poly-L-lysine coated coverslips at a concentration of ~2 × 10^6/ml at 4°C for 3 h. Immunocytochemistry was performed as previously described (Redgrove *et al.*, 2013) with primary antibodies used at concentrations of 1:50 (BAG6) and 1:100 (HSPA2) overnight at 4°C. Slides were viewed on an Olympus FV1000 confocal microscope with settings as previously indicated.

SDS-PAGE and immunoblotting

Lysates were prepared from non-capacitated and capacitated spermatozoa through SDS-extraction and resolved via SDS-PAGE or BN-PAGE as previously described (Redgrove *et al.*, 2011; Reid *et al.*, 2012). Human testis lysate (ab30257; Abcam, Cambridge, UK) and epididymis lysate (Genetex, Irvine, CA, USA) were boiled in the presence of β-mercaptoethanol and 10 μg of protein was loaded per lane. Both SDS-PAGE and BN-PAGE gels were transferred to nitrocellulose membrane using conventional western blotting techniques. Membranes were blocked in 3% w/v BSA in TBS supplemented with 0.1% polyoxyethylenesorbitan monolaurate (Tween-20; TBST) for 1 h at RT and rinsed with TBST prior to primary antibody incubation. Primary antibodies were diluted in 1% BSA/TBST and applied to membranes at concentrations of 1:500 (BAG6), 1:1000 (HSPA2 and ADAM30) and 1:4000 (Tubulin) overnight at 4°C under rotation. Membranes were washed (3 × 10 min) in TBST and appropriate HRP-conjugated secondary antibodies diluted in 1% BSA/TBST (anti-rabbit 1:1000; anti-mouse 1:5000) were applied for 1 h at RT under rotation. After further wash steps cross-reactive proteins were observed using an enhanced chemiluminescence kit (GE Healthcare) and viewed using a LAS-4000 detection system (Fujifilm, Tokyo, Japan). Protein-loading equivalency was confirmed using anti-tubulin antibodies as previously described (Bromfield *et al.*, 2013).

Duolink proximity ligation assay

In-situ proximity ligation assays were conducted according to manufacturer's instructions (OLINK Biosciences, Uppsala, Sweden; as described by Redgrove *et al.*, 2012) using anti-HSPA2 and anti-BAG6; or anti-HSPA2 and anti-tubulin or anti-BAG6 and anti-tubulin primary antibodies. Appropriate synthetic oligonucleotide-conjugated secondary antibodies (anti-rabbit for HSPA2 and anti-mouse for BAG6 and tubulin) were purchased from OLINK Biosciences. Coverslips were mounted as previously described for immunocytochemistry and visualized with an Avio Imager A1 fluorescence microscope (Carl Zeiss) and images were taken using an Olympus DP70 microscope camera. When target proteins reside within a maximum distance of 40 nm, this reaction results in the production of a discrete fluorescent signal that appears as a red spot. These signals were recorded as 'PLA positive' when a spermatozoon possessed three or more punctate fluorescent spots over the sperm head and in this way proximity ligation assay (PLA) fluorescence was quantified for 200 cells per slide with the percentage of 'PLA positive' spermatozoa being recorded for each sample.

Immunoprecipitation

Non-capacitated and capacitated spermatozoa were prepared as above. Cell lysis was performed on populations of ~100 × 10^6 cells from each treatment at 4°C for 2 h in lysis buffer consisting of 10 mM CHAPS, 10 mM HEPES, 137 mM NaCl and 10% glycerol with the addition of protease inhibitors (Roche). Lysis was completed with centrifugation at 14 000*g* at 4°C for 20 min. The cell lysates were then added to 50 μl aliquots of washed protein G Dynabeads (Life Technologies) and incubated under rotation to preclear at 4°C for 1 h. Anti-HSPA2 or anti-BAG6 antibodies at a concentration of 10 μg in 200 μl of PBS were conjugated to fresh aliquots of washed (supernatant removed) Dynabeads by incubation for 2 h at 4°C under rotation. Following antibody binding the cross-linking reagent, 3,3′-dithiobis[sulfosuccinimidyl-propionate] (DTSSP), was added at a final concentration of 2 mM and cross-linking was performed at RT for 30 min after which 20 mM TRIS was added to each tube for an additional 15 min at RT to quench the reaction. Beads were washed (3×) in 200 μl of lysis buffer and the wash supernatants were kept and stored at −20°C.

Immunoprecipitation was then performed by adding 1 ml of precleared lysate to HSPA2 or BAG6 antibody bound beads and incubating under rotation overnight at 4°C. After incubation, the supernatant was transferred to a clean tube and washed (3×) in 200 μl of PBS. Finally, the beads were resuspended in 100 μl of PBS and transferred to a fresh tube to avoid co-elution of proteins bound to the tube. Target antigen was eluted from the beads by boiling in the presence of SDS loading buffer (containing 8% β-mercaptoethanol). The same elution was performed on precleared beads (as well as 10 μl of fresh protein G beads for a bead-only control), and these solutions were loaded onto a NuSep 4–20% TRIS-Glycine gel for analysis via SDS-PAGE. In addition, antibody-only controls were prepared by loading 5 μl of anti-HSPA2 or anti-BAG6 in the presence of SDS loading buffer into appropriate gel lanes. The third wash of the antibody bound beads for each treatment was also loaded onto the gel after boiling in the presence of SDS loading buffer for 5 min. A duplicate gel was prepared for immunoblotting with reciprocal antibodies and both were resolved at 150 V for ~1 h. Electro-transfer of proteins was performed as previously described (Redgrove *et al.*, 2013) and proteins of interest were detected according to the western blotting procedures outlined above.

Blue native PAGE

Both non-capacitated and capacitated spermatozoa were prepared for blue native (BN)-PAGE as previously described (Redgrove *et al.*, 2012). Dialyzed native protein lysates were loaded onto blue native polyacrylamide gels (NativePAGE Novex 4–16%, Bis-Tris gels; Invitrogen) and resolved using a NativePAGE cathode and anode buffer (Redgrove *et al.*, 2011). Following retrieval from the cassettes, gels were either stained with Coomassie G250 or prepared for western blotting, as previously described.

Mass spectrometry and protein identification

Mass spectrometry (MS) analyses were performed at the Australian Proteome Analysis Facility using a one-dimensional (1D) nano-liquid chromatography electrospray ionization MS/MS interface, as previously described (Redgrove *et al.*, 2011).

Peptide data were exported in a format suitable for submission to the database search program, Mascot (Matrix Science Ltd, London, UK). Peaklists were searched against *Homo sapiens* in the SwissProt database (2013). High scores in the database search indicate a likely match, which was confirmed or qualified by operator inspection. Search results were generated with a significance threshold of $P < 0.02$ with a cut-off score of 34 for all

samples and a peptide mass tolerance of ±300 ppm, a fragment mass tolerance of ±0.6 Da, and maximum trypsin missed cleavages set to 1.

Statistics

Experiments reported on in this study were replicated at least three times using independent samples, and data are expressed as mean values ± SE where appropriate. Western blot band densitometry was performed relative to tubulin control using ImageJ analysis software (version 1.41o, National Institute of Health, USA) and was performed on three replicate blots. Statistical analysis was performed using a two-tailed unpaired Student's *T*-test with Microsoft Excel (Version 14.0.0). Differences were considered significant when $P < 0.05$.

Results

Detection of BAG6 and HSPA2 through western blotting

The presence of BAG6 and HSPA2 was verified in testis, epididymis and mature sperm lysate (Fig. 1A). In the case of BAG6, a band of the correct size (~120 kDa) was detected through immunoblotting with anti-BAG6 antibodies in both testis and epididymis lysates. Similarly, probing the lysate of mature spermatozoa (ejaculated spermatozoa that have been Percoll enriched) with anti-BAG6 also revealed the presence of a ~120 kDa band. Given that probing each of these western blots with anti-α-tubulin revealed equivalent protein loading, this result highlights the differential expression of BAG6, with three-fold more ($P = 0.02$) of the protein being detected in mature spermatozoa and epididymis lysate compared with testis lysate (Fig. 1B). Conversely, immunoblotting with anti-HSPA2 revealed statistically similar levels ($P > 0.05$) of the ~72 kDa protein in testis, epididymis and mature sperm lysate (Fig. 1C). In spermatozoa collected post-ejaculation, the presence of BAG6 was detected in both non-capacitated and capacitated spermatozoa through immunoblotting (Fig. 1D) with a ~120 kDa band detected in lysates probed with anti-BAG6 antibody. As expected, HSPA2 was also detected in both non-capacitated and capacitated human spermatozoa with equal protein loading established through the use of anti-α-tubulin antibodies; band densitometry analyses revealed no significant difference in BAG6 or HSPA2 expression levels between non-capacitated and capacitated sperm populations (Fig. 1E and F).

Immunolocalization of BAG6 and HSPA2 in developing human spermatozoa

After evaluating the presence of BAG6 and HSPA2 during testicular and post-testicular sperm maturation, we next sought to determine their localization within human testis and epididymis. For the purpose of this study, immunolocalization was performed on human testis and epididymis tissue sections purchased from Abcam, as this material displayed the best overall morphology of the various commercially sourced specimens examined (Supplementary Fig. S1). Nevertheless, due to the lack of germ cell organization within each tubule, conclusions regarding the expression of these proteins at specific germ cell stages could not be drawn. Instead these tissue sections were used to determine the presence/absence of BAG6 and evaluate the co-localization of BAG6 and HSPA2 in the developing germ cells.

Anti-HSPA2 immunohistochemistry revealed the presence of this protein in early and later stage germ cells of the testis with particularly strong expression in spermatids and testicular sperm situated within the lumen and limited expression observed in the interstitial tissue (Fig. 2A and E). BAG6 was detected in a majority of germ cells with strong co-localization of BAG6 and HSPA2 observed in a majority of early (spermatogonia-like) and later (spermatocyte-like) stage germ cells (Fig. 2B and F). Importantly, in our anti-mouse and anti-rabbit secondary only controls, only minimal background fluorescence could be detected (Supplementary Fig. S2A).

Immunolocalization of BAG6 and HSPA2 in the human epididymis revealed the presence of both chaperones in epididymal spermatozoa (Fig. 2I–L). The co-localization of these proteins in spermatozoa was observed in all regions and did not appear to be dependent upon the maturational status of the cells. The presence of HSPA2 and BAG6 was also consistently detected in the epithelial cells of the epididymal tubule (Fig. 2I–P). Again, minimal background fluorescence was observed in our secondary only controls (Supplementary Fig. 2B). Given these results, we were interested in evaluating the distribution profile and nature of the HSPA2/BAG6 interaction in spermatozoa collected after ejaculation.

Under non-capacitating conditions, immunocytochemistry revealed BAG6 localized predominantly to the equatorial region of the sperm head with labeling also present in the neck/connecting piece of the cell (Fig. 3A). As previously recorded within these cells (Redgrove *et al.*, 2012), HSPA2 was found in the anterior region of the sperm head with an accompanying band of bright fluorescence within the equatorial region. Accordingly, co-localization of the two proteins occurred exclusively in the equatorial segment of non-capacitated sperm.

Similar labeling patterns were observed for both BAG6 and HSPA2 in capacitated spermatozoa (Fig. 3B). However, in capacitated cells, additional BAG6 labeling was observed extending through the anterior domain of the sperm head resulting in strong co-localization with HSPA2 across this region. This labeling pattern was quantified, by recording the percentage of spermatozoa that stained positively for BAG6 or HSPA2 across both the equatorial and anterior sperm head (Fig. 3C), revealing a 4-fold increase in the number of spermatozoa possessing BAG6 labeling in the anterior domain of the sperm head after capacitation.

Evidence for interaction between BAG6 and HSPA2 in mature spermatozoa

Given the evidence of co-localization between BAG6 and HSPA2 throughout human sperm development in the testis and epididymis, it seems that an association between these proteins may be established in the early stages of germ cell development, as has been previously shown in the mouse (Sasaki *et al.*, 2008), and upheld throughout maturation in the male and female reproductive tracts. Although we were unable to follow this interaction further in the testis or epididymis due to a lack of accessible biological material, the remaining series of experiments sought to characterize the interaction between BAG6 and HSPA2 in mature spermatozoa.

Using an in-situ PLA, the potential association between BAG6 and HSPA2 was examined in mature human spermatozoa (Fig. 4A). A threshold level of ≥3 punctate red fluorescent spots located within the sperm head was set for positive PLA labeling and the percentage of non-capacitated and capacitated spermatozoa displaying these signals was recorded (Fig. 4B). In ~85% of non-capacitated spermatozoa, PLA

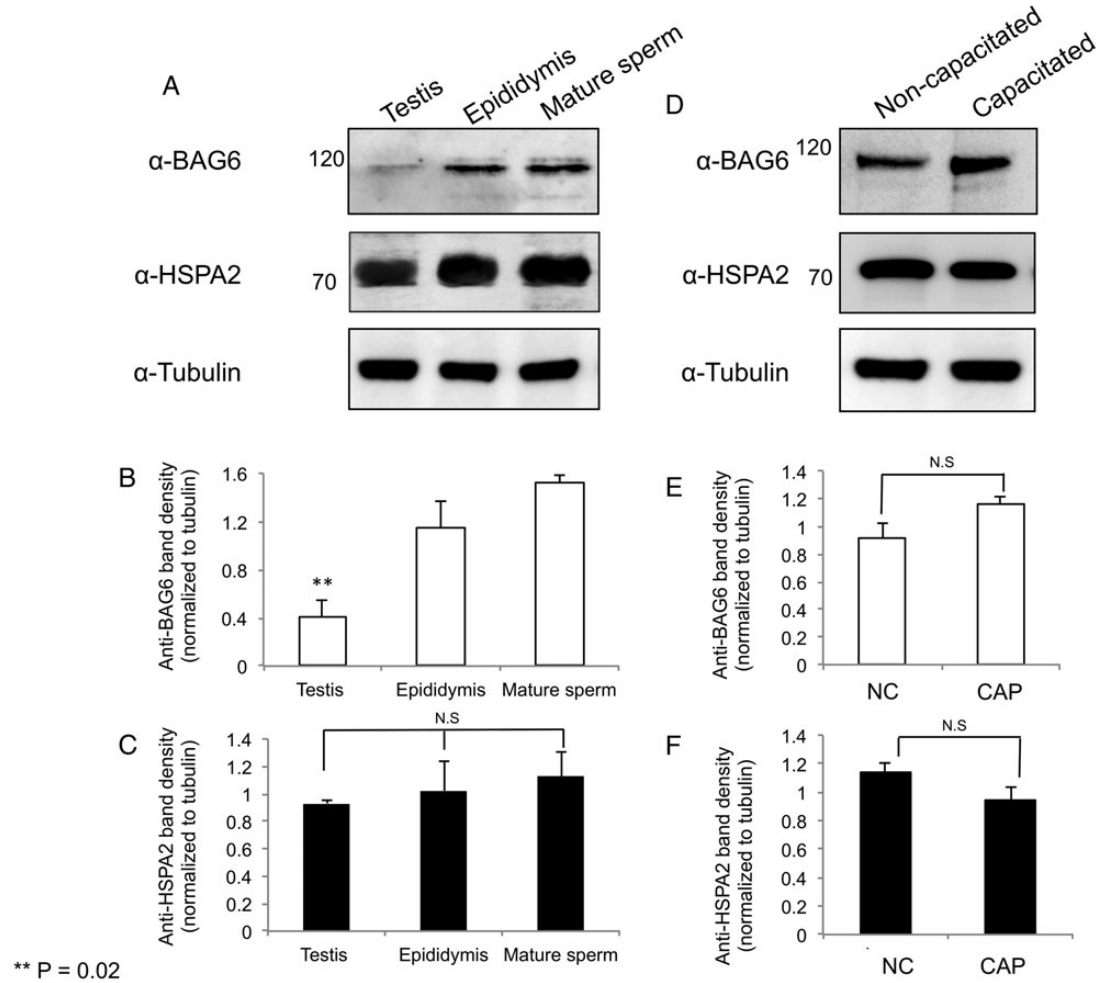

Figure 1 Confirmation of BAG6 and HSPA2 expression through immunoblotting analyses. (**A**) Commercially acquired human testis and epididymis lysates were submitted to SDS-PAGE alongside a mature sperm lysate control and transferred to nitrocellulose membrane for immunoblotting procedures. Separate membranes were probed with anti-BAG6 and anti-HSPA2 antibodies and corresponding secondary antibodies, and cross-reactive proteins were visualized using enhanced chemiluminescence. Densitometry analyses were performed from three replicate western blots for both BAG6 (**B**) and HSPA2 (**C**) and results were plotted relative to anti-tubulin controls. (**D**) Non-capacitated and capacitated sperm populations were lysed using SDS-extraction procedures, and extracted protein was also subjected to SDS-PAGE and immunoblotting with antibodies to BAG6 and HSPA2. Band densitometry analyses were performed for both BAG6 (**E**) and HSPA2 (**F**) relative to tubulin controls. $^{**}P = 0.02$. N.S = not significant, $P > 0.05$.

fluorescence was observed predominantly over the equatorial and post-acrosomal region of the sperm head (Fig. 4A and B). In contrast, an equivalent number of the capacitated sperm population (~90%) displayed PLA fluorescence primarily over the apical ridge of the sperm head with some labeling of the equatorial and post-acrosomal region. The specificity of the PLA signal was confirmed through the use of an irrelevant antibody (anti-α-tubulin) in combination with anti-BAG6 or anti-HSPA2 antibodies (Fig. 4C).

As these data suggest that BAG6 and HSPA2 are in close association in mature human spermatozoa, we next sought to confirm this putative interaction using a reciprocal co-immunoprecipitation approach whereby lysates were precipitated with either anti-BAG6 or anti-HSPA2 antibodies and the eluates were tested for the presence of each target protein. As shown in Figure 6, this strategy proved effective in isolating both bait proteins along with that of their corresponding targets. Indeed, a band of the appropriate size for BAG6 (~120 kDa) was detected in the anti-HSPA2 immunoprecipitation eluate (Fig. 4E) and similarly, the ~72 kDa HSPA2 band was detected in the reciprocal BAG6 immunoprecipitation eluate (Fig. 4G). The specificity of each IP was verified through the inclusion of antibody-only and bead-only controls in addition to the preclear bead eluate and the material recovered after washing the beads prior to elution (Fig. 4D–G).

After confirming the interaction between BAG6 and HSPA2 in mature spermatozoa, we next sought to determine whether these proteins are implicated in the formation of multimeric protein complex(es) in human spermatozoa. For this purpose, we employed blue native-PAGE, a technique that we have previously used to demonstrate that HSPA2 partitions into a number of high molecular weight protein complexes, the best characterized of which has an aggregate molecular weight of ~200 kDa (Redgrove *et al.*, 2012). In this study, in excess of 10

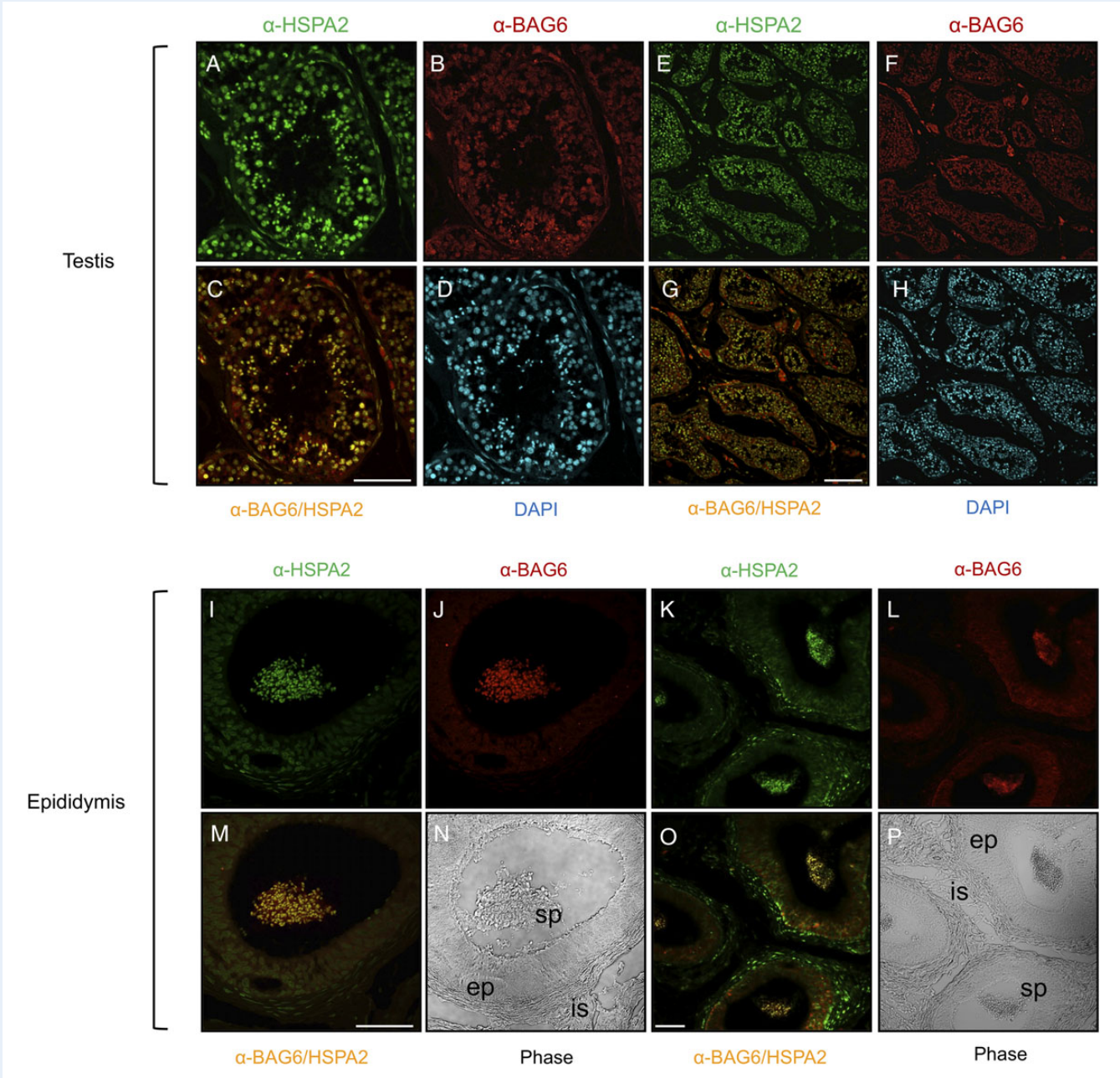

Figure 2 Detection of BAG6 within the human testis and epididymis. (**A–H**) Immunohistochemistry was performed on human testis tissue using antibodies against BAG6 (red) and HSPA2 (green) and DAPI counterstain (blue). Confocal microscopy images were taken using either a ×60 (A–D) or a ×20 objective (E–H). Co-localization is most distinct in the later stage germ cells (spermatocytes, developing spermatids and spermatozoa). DAPI nuclear staining (D and H) reveals that morphology is quite abnormal in some tubules (H) and often these abnormal tubules lack elongating spermatids and sperm. Scale bar = 100 μm (A–D) and 1 mm (E–H). (**I–P**) Immunohistochemistry performed with anti-HSPA2 antibody revealed the concentrated expression of this protein in epididymal spermatozoa (sp) residing in the lumen (l). Additionally, HSPA2 was detected in principal cells (shown clearly in I) of the epithelium (ep) (N). BAG6 expression mirrored that of HSPA2 in epididymal spermatozoa (J) and was also detected in the principal cells of the epithelium (L). Images M and O depict the strong co-localization observed between HSPA2 and BAG6 proteins (yellow) in these merged images. These labeling patterns were not region specific, and images are representative of all sections used. Phase contrast images are included to illustrate epididymal morphology, and all images were taken on a confocal microscope using either a ×60 (I–L) or a ×40 objective (M–P). Scale bars = 100 μm.

protein complexes of aggregate molecular weight >150 kDa were successfully resolved through the application of BN-PAGE (Fig. 5A). Immunoblotting with antibodies against HSPA2 and BAG6 revealed the presence of these two proteins in one dominant complex of ~280 kDa (Fig. 5A), although both proteins were also represented separately in a number of additional complexes (Fig. 5A). Finally, the presence of HSPA2 and BAG6 within the 280 kDa complex was confirmed through MS. Twelve unique peptides corresponding to HSPA2 were

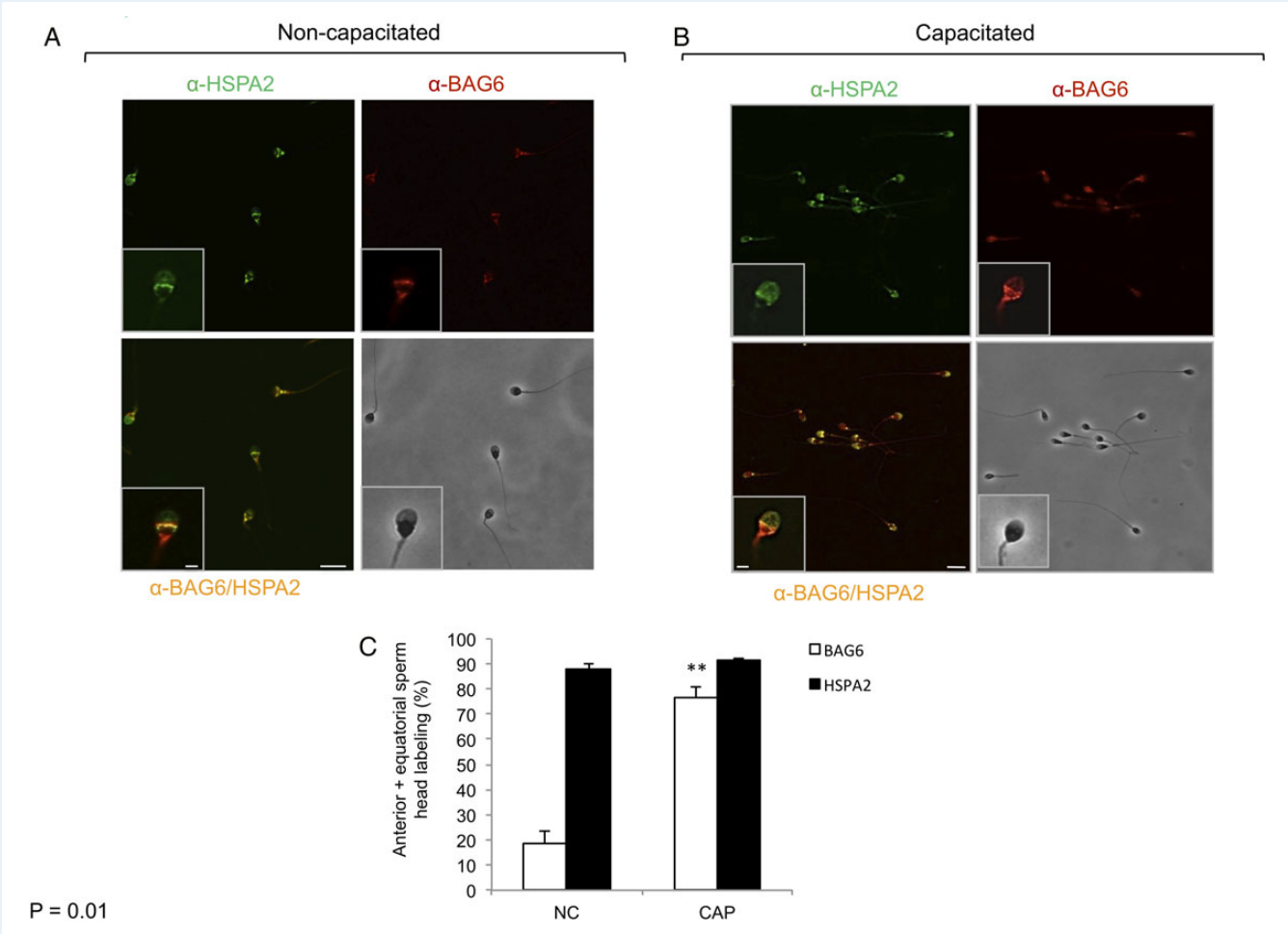

Figure 3 Immunolocalization of BAG6 and HSPA2 in non-capacitated and capacitated human spermatozoa. Immunocytochemistry was performed with anti-HSPA2 and anti-BAG6 antibodies on spermatozoa that had been either held in a non-capacitated state (**A**) in a modified BWW media lacking $NaHCO_3^-$ or driven to capacitate (**B**) in BWW supplemented with 25 mM $NaHCO_3^-$, 3 mM ptx and 5 mM dbcAMP. Labeling patterns were quantified by recording the percentage of spermatozoa that stained positively for BAG6 or HSPA2 across both the equatorial and anterior sperm head with 100 cells examined per slide (**C**). Confocal images were taken using a ×60 objective. $^{**}P < 0.01$ between NC anti-BAG6 labeling and CAP anti-BAG6 labeling. Scale bars = 3 μm for inset images and 10 μm for large images.

detected with an overall MASCOT score of 695 (Table I). Although BAG6 identification was based on a single unique peptide, this peptide returned a MASCOT score of 127 and did not map to other BAG-family domain proteins. Given that the aggregate molecular weight of BAG6 and HSPA2 is only 192 kDa, it is considered unlikely that they are the sole constituents of the 280 kDa complex. Rather, MS analysis of the 280 kDa Blue Native gel plug identified two unique peptides corresponding to the 89 kDa A Disintegrin And Metalloprotease 30 protein (ADAM30). Upon probing BN-PAGE blots of non-capacitated and capacitated native sperm lysate with anti-ADAM30, a band of 280 kDa was detected that co-migrated with that detected by anti-BAG6 and anti-HSPA2, thus raising the possibility that ADAM30 may be present within the same complex as our chaperone proteins of interest (Fig. 5B). The specificity of this antibody was confirmed using non-capacitated and capacitated human sperm SDS lysates subjected to SDS-PAGE and immunoblotted with anti-ADAM30 (Supplementary Fig. S2).

Assessment of BAG6 protein expression in the spermatozoa of infertile men

As BAG6 is a regulator of HSPA2 stability in mouse germ cells (Sasaki *et al.*, 2008) and our results support the interaction of these proteins throughout human germ cell development, we were interested in determining whether the lack of HSPA2 in human spermatozoa that are unable to bind to homologous zona pellucida (Redgrove *et al.*, 2013) may reflect a deficiency in BAG6 protein expression levels. For this purpose, a preliminary study was conducted using patients selected on the basis of failed zona pellucida adherence after overnight incubation with homologous oocytes. When sperm lysates from these patients were subjected to immunoblotting, they were found to be deficient in both HSPA2 and BAG6 compared with fertile controls (Fig. 6). Subsequent interrogation of these lysates with anti-tubulin confirmed equivalent protein loading (Fig. 6C). While this study remains to be expanded, this result provides impetus for further study into BAG6 as a regulator of HSPA2 stability and sperm function.

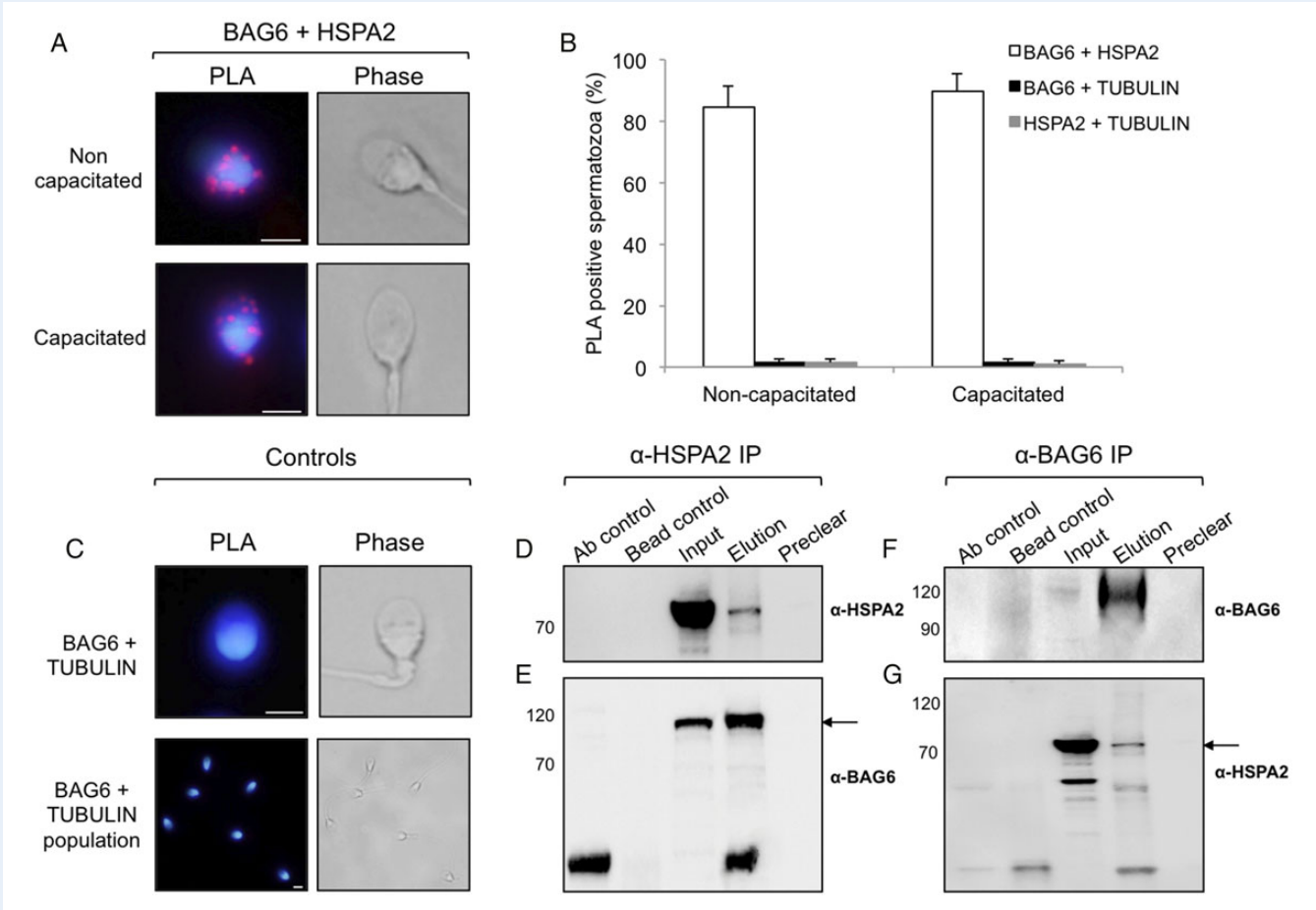

Figure 4 Analysis of BAG6/HSPA2 interaction in human spermatozoa. (**A–C**) A proximity ligation assay was used to confirm the putative interaction between BAG6 and HSPA2 in human spermatozoa. This assay results in the production of punctate red fluorescent signals when proteins of interest reside within a maximum of 40 nm from each other. For clarity, spermatozoa were counterstained with DAPI. As shown in (A), positive PLA signals appearing as a number of discrete red fluorescent spots were detected in the head of both non-capacitated and capacitated sperm populations. (B) Quantification of the percentage of 'PLA positive' cells (those cells possessing ≥ 3 punctate fluorescent spots over the sperm head) was performed revealing that HSPA2/BAG6 were closely apposed in $>80\%$ of both non-capacitated and capacitated spermatozoa. Importantly, by employing anti-tubulin as an irrelevant antibody control (C) we were able to confirm the specificity of this assay with $<5\%$ of the sperm population displaying positive PLA signals in the presence of BAG6 and tubulin antibodies. Scale bars = 3 μm. (**D–G**) Confirmation of HSPA2/BAG6 interaction in capacitated spermatozoa was sought using a reciprocal co-immunoprecipitation strategy in which both HSPA2 and BAG6 were used as bait to pull down interacting partners. The captured sperm proteins were eluted from protein G beads and resolved on SDS-PAGE gels alongside an antibody-only control (Ab control), a bead-only control (Bead control), capacitated sperm lysate (Input) and a precleared control (preclear). (D) HSPA2 IP blots were probed with anti-HSPA2, and the specificity of the IP was confirmed through the detection of a 72 kDa band in both the elution and input lanes but importantly not in the control lanes. Probing these blots with anti-BAG6 (E) revealed the presence of BAG6 both in the IP eluate at 120 kDa and in the input lysate lane (denoted by an arrowhead) but again not in the control lanes. While an additional low molecular weight band was detected in the eluate, this band corresponded to that detected in the antibody control and was deemed to be antibody contamination produced during the final elution step. (F) Confirmation of the efficacy of the reciprocal IP (performed with anti-BAG6) was achieved by the detection of the 120 kDa band corresponding to BAG6 in the elution and input lanes but not the control lanes. (G) When the BAG6 IP blot was probed with anti-HSPA2, the presence of this protein at 72 kDa was detected in the eluate and the input lanes (denoted by an arrow) with lower molecular weight bands aligning with those of the antibody control lanes and likely to be a small amount of antibody contamination. This assay indicated that both BAG6 and HSPA2 could be co-immunoprecipitated from capacitated human spermatozoa.

Discussion

While the function of HSPA2 in human spermatozoa has been evaluated through recent studies focusing on the importance of this chaperone in sperm–oocyte interactions (Redgrove *et al.*, 2012, 2013), the mechanisms regulating its expression in cases of idiopathic male infertility are yet to be fully resolved. Potential mechanisms involving mutations, polymorphisms and/or aberrant methylation status of the *Hspa2* gene are currently under consideration (Nixon *et al.*, 2015). However, given the complete absence of mature spermatozoa resulting from deletion of the *Hspa2* gene in the mouse model and the varying levels of HSPA2 protein-deficiency observed in the spermatozoa of men with idiopathic infertility, alternative mechanisms that result in the post-

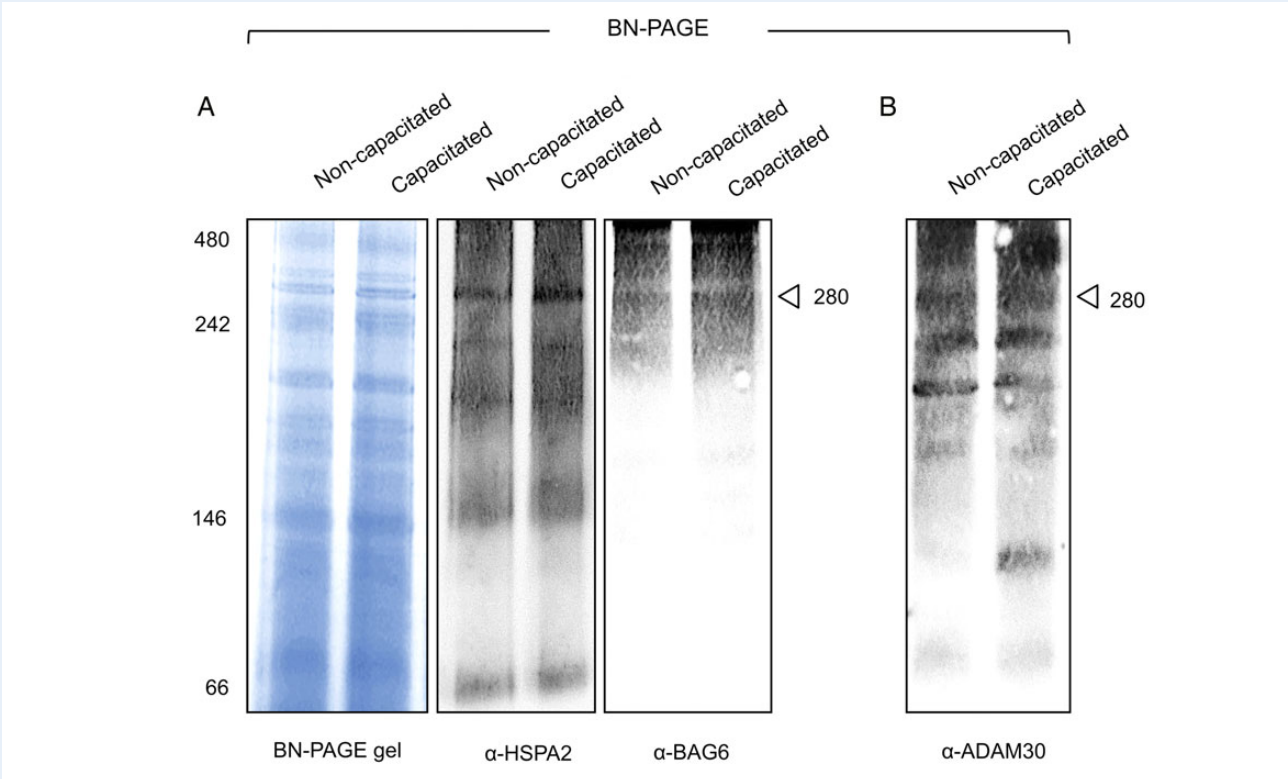

Figure 5 Detection of BAG6 and HSPA2 complex(es) in native lysates of human spermatozoa. (**A**) Non-capacitated and capacitated sperm were subjected to native protein lysis, and the resulting protein complexes were resolved and visualized by Coomassie staining or prepared for western blotting. Coomassie staining revealed the presence of several protein complexes in both non-capacitated and capacitated human sperm while immunoblotting with anti-HSPA2 resulted in the detection of its presence in five key protein complexes of aggregate molecular weights >100 kDa. Probing of corresponding blots with anti-BAG6 revealed its presence in several complexes, the most dominant of which had an aggregate molecular weight of 280 kDa and co-migrated with a similar complex detected by anti-HSPA2 antibodies (denoted by an arrowhead). 1D nano-LC/MS analysis of the 280 kDa complex confirmed the presence of HSPA2 and BAG6 in additional to revealing the presence of ADAM30 within this complex. (**B**) The presence of ADAM30 in this complex was subsequently confirmed by immunoblotting of native sperm lysates with anti-ADAM30 antibodies, resulting in the detection of this protein in a band of 280 kDa in addition to several other high molecular weight complexes (~140 to ~480 kDa).

translational regulation of this protein have become an important focus of our studies. Specifically this work has sought to characterize the expression of the co-chaperone BAG6 that has been implicated in maintaining the stability of HSPA2 by protecting the protein from polyubiquitylation and subsequent destruction (Sasaki *et al.*, 2008). These analyses have established that BAG6 and HSPA2 share an overlapping pattern of spatial and temporal expression within developing human testicular germ cells. Moreover, to the best of our knowledge, we have provided the first evidence that BAG6 is readily incorporated, and perhaps even preferentially sequestered, into the spermatozoa that leave the testes. The potential importance of this finding for regulation of HSPA2 stability is highlighted by several lines of evidence that BAG6 and HSPA2 not only form a stable interaction within the head of human spermatozoa but that the distribution of this complex is dynamically influenced by the capacitation status of these cells.

Previous work on BAG6 has established that it interacts directly with the ATPase domain of HSPA2 and other HSP70 family members via electrostatic and hydrophobic docking of its BAG domain (Sasaki *et al.*, 2008). Such interactions promote substrate release from HSP70 proteins, thus allowing for specification and/or diversification of HSP70 chaperone functions (Takeyama *et al.*, 1999). These functions include the HSP70-mediated protein refolding and ubiquitin-mediated proteolysis of misfolded proteins in the mouse testis and appear to be common to a variety of cell types (Kabbage and Dickman, 2008; Corduan *et al.*, 2009; Minami *et al.*, 2010). It is therefore tempting to speculate that BAG6 may fulfill analogous functions in the human testes. Support for this assertion rests with the overlapping expression of HSPA2 and BAG6 that we observed in testicular tissue and the high degree of sequence homology between human and mouse BAG6 proteins (89% amino acid sequence identity).

The role of the BAG6/HSPA2 interaction that we observed in both the epididymal epithelium and in maturing spermatozoa within the lumen of this organ remains less certain. Interestingly, the localization of HSPA2 to epididymal spermatozoa in our study diverges from observations of the mouse epididymis where the protein is purportedly shed along with the cytoplasmic droplet during sperm transit from the caput to caudal regions of the duct (Ijiri *et al.*, 2011). However, our detection of HSPA2 in the epithelial cells of the epididymis reflects that of previous localization studies (Legare *et al.*, 2004). The expression of BAG6 in the human epididymis has not previously been reported and

Table I Identification of BAG6, HSPA2 and ADAM30 in a human sperm protein complex.

Protein (symbol)	UniProt accession number	Mw (kDa)	No. pepetides Matched	Peptide sequences	MASCOT score for individual peptides	Overall MASCOT score
Heat Shock 70 kDa protein 2 (HSP72)	P54652	70	13	K.FDLTGIPPAPR.G R.FEELNADLFR.G K.NALESYTYNIK.Q K.CQEVINWLDR.N K.GQIQEIVLVGGSTR.I R.TTPSYVAFTDTER.L K.TFFPEEISSMVLTK.M K.TFFPEEISSMVLTK.M R.IINEPTAAAIAYGLDK.K K.NQVAMNPTNTIFDAK.R R.IINEPTAAAIAYGLDKK.G K.VHSAVITVPAYFNDSQR.Q K.LYQGGPGGGSGGGGSGASG GPTIEEVD.-	49 80 82 56 101 101 (93) 96 124 46 81 97 77	695
Large proline-rich protein BAG6 (BAG6)	P46379	119	1	R.LLGNTFVALSDLR.C	127	127
Disintegrin and Metalloproteinase domain-containing protein 30 (ADAM30)	Q9UKF2	89	2	K.ALEVWTDFNK.I R.VGYPELAEVLGR.F	75 92	136

MS analysis resulted in the detection of 12 unique peptides corresponding to human Heat Shock 70 kDa protein 2 (HSPA2) from in-gel tryptic digestion of the 280 kDa BN-PAGE gel band resolved from capacitated human sperm native lysate. These peptides had an overall mascot score of 695. One unique peptide corresponding to human large proline-rich protein BAG6 (BAG6) was detected from the same gel band with a mascot score of 127 and two unique peptides corresponding to Disintegrin and Metalloproteinase domain-containing protein 30 (ADAM30) were detected (also from this 280 kDa band) with a combined mascot score of 136.

similarly, a putative function for BAG6 in the male mouse reproductive tract has not been explored as *Bat3*$^{-/-}$ (*Bag6*) mice are characterized by a complete absence of epididymal spermatozoa owing to arrested spermatogenesis (Sasaki *et al.*, 2008). It could be posited that the detection of BAG6 in the epithelium, while unexpected, may relate to its ability to bind the hydrophobic region of misfolded proteins to direct their refolding or removal in the prevention protein aggregation (Payapilly and High, 2014; Wunderley *et al.*, 2014). In this context, it is well known that the luminal protein content of the epididymis is uniquely susceptible to aggregation due to its high macromolecular and low water content (Cornwall, 2009). Indeed, such an environment has been shown to promote the formation of protein aggregates and/or amyloid structures where chaperones such as clusterin attend to protein quality control through their ability to interact with hydrophobic proteins and maintain their solubility (Cornwall *et al.*, 2007; Cornwall, 2009).

In mature spermatozoa, BAG6 underwent a conspicuous relocation or unmasking event following the induction of capacitation, such that an additional pool of immunoreactive protein was detected in the anterior region of the head. This event coincided with the appearance of strong co-localization with HSPA2 in both the equatorial and apical regions of the capacitated sperm head.

The marked dispersal of BAG6 expression over the apical region of the sperm head during capacitation is closely tied to HSPA2 localization and may be indicative of a moonlighting role for BAG6 in the regulation of HSPA2 function in mature sperm or perhaps as an independent molecular chaperone involved in the critical process of sperm surface remodeling that occurs prior to sperm–oocyte adhesion and fusion processes (Gadella *et al.*, 2008). This change in location also reflects our previously reported data on the translocation of proteins within membrane rafts (Nixon *et al.*, 2011), an event that results in the reshuffling of molecules toward the apical region of the sperm head during capacitation. While HSPA2 is known to be present within such human sperm membrane microdomains (Nixon *et al.*, 2011), it remains to be established whether this mechanism is responsible for the change in location of BAG6.

In this study, support for an interaction between BAG6 and HSPA2 in human spermatozoa was demonstrated through the co-elution and co-migration of these proteins using immunoprecipitation and BN-PAGE analyses, respectively. Certainly the partitioning of BAG6 into sperm protein complexes is highly typical of the action of this protein in other mammalian cells where it has been shown to form complexes with ubiquitin-like 4a (Ubl4a) and transmembrane domain recognition complex 35 (TRC35) to carry out its roles in protein homeostasis through the mislocalized protein and endoplasmic reticulum-associated degradation pathways (Hessa *et al.*, 2011; Mock *et al.*, 2015). Interestingly, the stability of UBL4a is governed by BAG6 binding, and depletion of the BAG6 complex impairs the efficient ubiquitination of mislocalized proteins, providing yet another example of the importance of BAG6 in regulation of post-translational protein stability (Hessa *et al.*, 2011).

Given that BAG6 is important for ubiquitin-mediated metabolism of newly synthesized polypeptides but spermatozoa themselves are thought to possess a limited intrinsic system of proteolysis and are relatively ill equipped for protein synthesis after leaving the testis (Wykes *et al.*, 1997), it is curious that this protein would be carried over into mature spermatozoa. Despite this, the presence of BAG6 in the

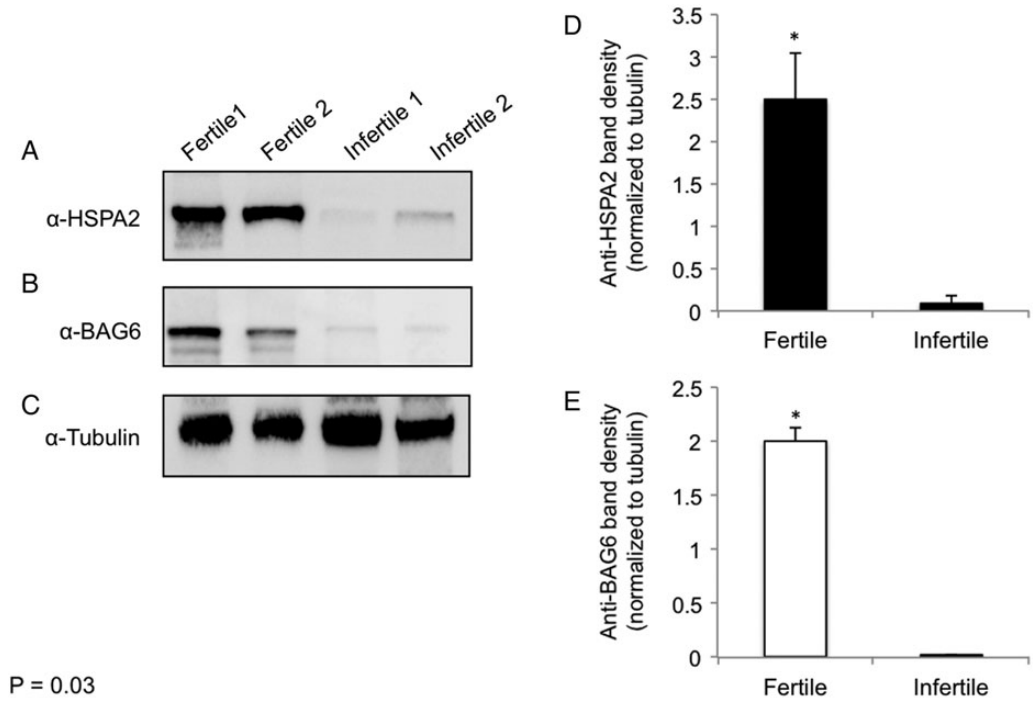

Figure 6 Analysis of BAG6 protein expression in the spermatozoa of infertile men. Infertile male IVF patients were selected based on a complete failure of sperm–zona pellucida binding following standard IVF. The spermatozoa from these patients were subjected to SDS-extraction and lysates were resolved alongside known fertile sperm lysate controls on SDS gels before being transferred to nitrocellulose membranes for immunoblotting. (**A**) Probing of these blots with anti-HSPA2 revealed a marked under-representation of the HSPA2 protein in the infertile sperm lysates (Infertile 1 and 2). (**B**) Probing of equivalent blots with anti-BAG6 revealed the concomitant loss of the 120 kDa protein band corresponding to BAG6 in the same patients (Infertile 1 and 2). Importantly equal protein loading was confirmed by incubating each blot with an anti-tubulin antibody (**C**). Analyses of anti-HSPA2 (**D**) and anti-BAG6 (**E**) band density were performed (relative to tubulin) using ImageJ. *$P = 0.03$.

proteome of mouse and rat spermatozoa has been noted in several publications (Baker *et al*., 2008a, b; Dorus *et al*., 2012).

BAG6 was initially identified as a novel gene product encoded within the class III domain of the human major histocompatibility complex (MHC; Minami *et al*., 2010) and in the sperm proteome, it is just one of several immunity proteins (such as complement proteins CD46, CD55, CD59 and non-complement immunity proteins angiotensin-converting enzyme [ACE] and IZUMO1) that are beginning to attract attention as our understanding of complement immunity between spermatozoa, seminal plasma and the uterine epithelium develops (Inoue *et al*., 2005, 2010; Wira *et al*., 2005; Dorus *et al*., 2012).

A selection of these immunity proteins have acquired specific functionality in spermatozoa throughout evolution and in some cases have become enriched or specifically expressed in the testis (Dorus *et al*., 2012). For example, two isozymes of ACE exist, somatic ACE (sACE) and the testis-specific germinal ACE (gACE [or tACE]), in human tissues that are expressed by different promoters but encoded by the same gene. The latter of these isozymes has a unique N-terminal sequence and possesses both the well-characterized dipeptidase activity of sACE (Hooper, 1991; Esther *et al*., 1996) and GPIase activity that has led to its implication in the cleavage of glycosyl-phosphatidylinositol (GPI)-anchored proteins at the sperm surface prior to zona pellucida binding (Kondoh *et al*., 2005). Importantly, the cleavage of such molecules may allow for the correct positioning or 'unmasking' of zona pellucida receptors during the later stages of capacitation. Additionally, IZUMO1, an immunoglobulin superfamily protein, plays a significant role in sperm–oocyte fusion where it is capable of interacting with another immunity protein, CD9, on the oocyte surface; these functions completely diverge from the role of these proteins as regulators of the immune response (Miyado *et al*., 2000; Inoue *et al*., 2005, 2010). Many sperm proteins, particularly those involved in cell–cell interaction, are in fact immunity-related proteins and their functional development appears to be directed by their pleiotropic roles in both systems (Dorus *et al*., 2012). In this way, BAG6 may play a previously uncharacterized pleiotropic role in mature spermatozoa.

For example, the presence of BAG6 in mature human spermatozoa could suggest a role for this protein as a regulator of the truncated apoptotic cascade that has been characterized in spermatozoa (reviewed by Aitken, 2011). In line with this, BAG6 has been shown to be important for the acetylation of p53 in response to DNA damage in embryonic fibroblasts and other cell types (Sasaki *et al*., 2007). While the inclusion of BAG6 in this pathway requires explicit analysis in mammalian sperm, BCL-2 is known to be a key participant in germ cell apoptosis (by Koji and Hishikawa, 2003) and BAG6 has been shown to promote BCL-2 function in some cell types (Shaha *et al*., 2010). In addition, BAG6 itself is cleaved by caspase-3, another important molecule in the apoptotic

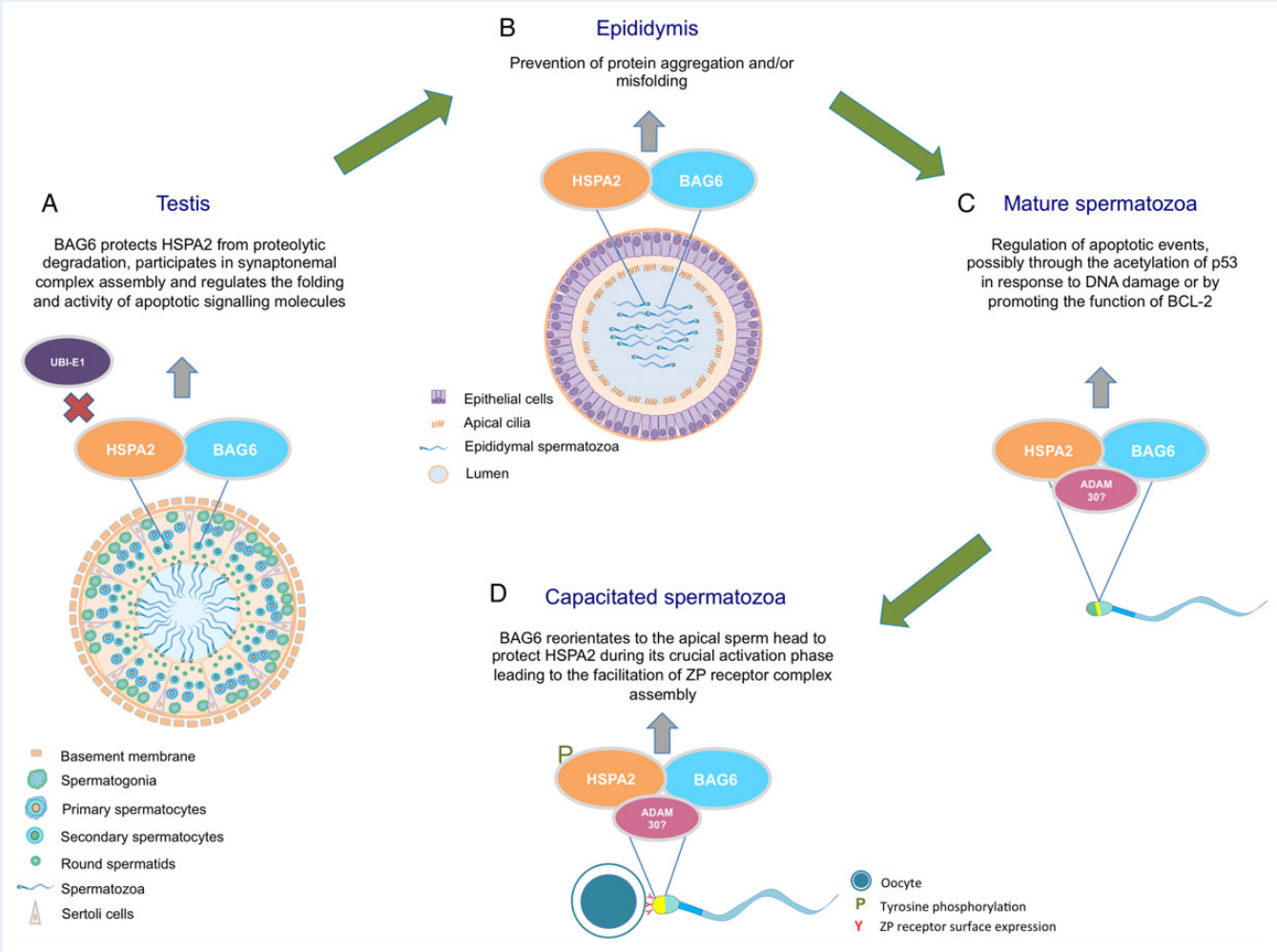

Figure 7 Putative functions of BAG6 throughout human sperm maturation. (**A**) Within the mouse testis, BAG6 and HSPA2 are involved in the coordination of synaptonemal complex assembly during meiosis and promote ubiquitin-mediated degradation of nascent chain polypeptides and the folding and activity of apoptotic signaling molecules (Kawahara *et al.*, 2013). While these roles are yet to be verified in the human, our co-localization studies suggest that these proteins are in close association within testicular germ cells and we postulate that these functions may be conserved in the human. In addition, the presence of BAG6 in the mouse testis has been shown to prevent the ubiquitin-dependent degradation of HSPA2 (Sasaki *et al.*, 2008). Our preliminary results suggest that the presence of BAG6 may also be important for the stability of HSPA2 in human germ cells. (**B**) In the epididymis, BAG6 and HSPA2 are both present in maturing spermatozoa. Additionally, localization of BAG6 to the epithelial cells of this duct may reflect its ability to bind the hydrophobic region of misfolded proteins and direct their refolding or removal to prevent protein aggregation. (**C**) In mature spermatozoa, BAG6 may act as a positive or negative regulator of the truncated apoptotic cascade that has been characterized in human spermatozoa. BAG6 is important for the acetylation of p53 in response to DNA damage in some cell types (Sasaki *et al.*, 2007) and can also promote the function of BCL-2 (B-cell lymphoma 2), a key participant in germ cell apoptosis (Koji and Hishikawa, 2003). (**D**) During sperm capacitation, a change in BAG6 localization was revealed such that an additional pool of immunoreactive protein was detected in the apical sperm head. This relocation or unmasking event corresponds temporally with the activation of HSPA2 and leads us to postulate that the presence of BAG6 is important in the prevention of protein misfolding or mislocalization events during the assembly of HSPA2-laden zona pellucida-receptor complexes (Redgrove *et al.*, 2012, 2013). In this way the presence of BAG6 may be critical for not only the stability of HSPA2 during testicular sperm maturation but also to maintain the function of this chaperone during sperm surface remodeling prior to zona pellucida interaction.

pathway (Wagenknecht *et al.*, 1999). Conversely, BAG6 can operate as an anti-apoptotic protein by interacting with apoptotic regulators such as PXT1, relocating them from the cytoplasm to the nucleus, thus protecting cells from PXT1-induced apoptosis (Kaczmarek *et al.*, 2011).

Although not the focus of these studies, the discovery of ADAM30 in complex with HSPA2 and BAG6 is intriguing as ADAM proteins have been documented to interact with chaperones during testicular and post-testicular maturation, to fulfill their various functions in the fertilization cascade (reviewed by Bromfield and Nixon, 2013). Although no ADAM protein has been shown to be singularly essential for sperm–oocyte interaction, an extensive body of literature suggests that the coordinated action of ADAMs with other proteins, such as the fusogen angiotensin-converting enzyme 3 (ACE3; Krege *et al.*, 1995), is vital for sperm–oocyte adhesion and fusion processes (Primakoff *et al.*, 1987; Cho *et al.*, 1998; Nishimura *et al.*, 2001; Choi *et al.*, 2003; Ellerman *et al.*, 2009; reviewed by Bromfield and Nixon, 2013). Notably, gene

knockout studies have demonstrated that *Adam3*$^{-/-}$ male mice are infertile as mutant spermatozoa are unable to navigate the uterotubal junction *in vivo* and are unable to participate in interactions with the zona pellucida *in vitro* (Muro and Okabe, 2011)

Despite its importance in murine fertilization, *Adam3* is a pseudogene in humans. Given there are in excess of 21 ADAM family members that have been detected in humans and seven of these, including ADAM30, are testis-specific, ADAM30 may be a good candidate for further analysis as a substitute for ADAM3. Given the cooperative function of BAG6 and HSPA2 in the processing of polypeptides, future studies will focus on confirming the interaction between ADAM30, HSPA2 and BAG6 and investigating potential ties between the protein-folding functions of these chaperones and the quality control of ADAM30 in human germ cells.

In view of the findings described in this study, we propose that BAG6 is a likely regulator of HSPA2 stability/function in human germ cells (Fig. 7). However, it is acknowledged that specific mechanistic studies involving the inhibition of BAG6 need to be performed to confirm this hypothesis. Excitingly, our preliminary studies evaluating protein deficiency in the patient population has revealed that spermatozoa that lack the ability to interact with homologous human zona pellucida associated with dysregulation of HSPA2 protein expression also have a severe deficiency in BAG6 protein expression compared with fertile controls. These data support the proposal by Sasaki and colleagues that BAG6 may be a new target for idiopathic male infertility and that the absence of BAG6 may lead to the accelerated degradation of HSPA2 in human germ cells either via a proteasomal (Sasaki *et al.*, 2008) or a lysosomal pathway (Sebti *et al.*, 2014). As the depletion of BAG6 is associated with the decreased stability of HSPA2 in other cell types, including mouse embryonic fibroblasts and human teratocarcinoma cells (Sasaki *et al.*, 2007), a coordinated role for BAG-family and heat shock protein family proteins may represent a highly conserved mechanism for nascent peptide control in biological systems.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

E.B. conceived the study, conducted the experiments and generated the manuscript. R.J.A. contributed to study design and data interpretation and B.N. contributed to study design, data interpretation and manuscript preparation and editing.

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Conflict of interest

None declared.

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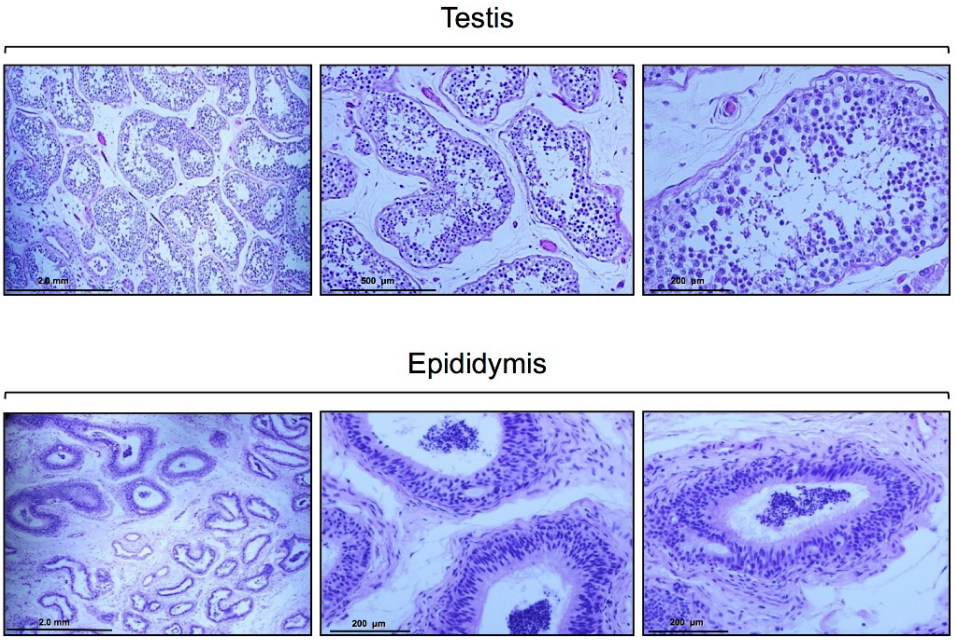
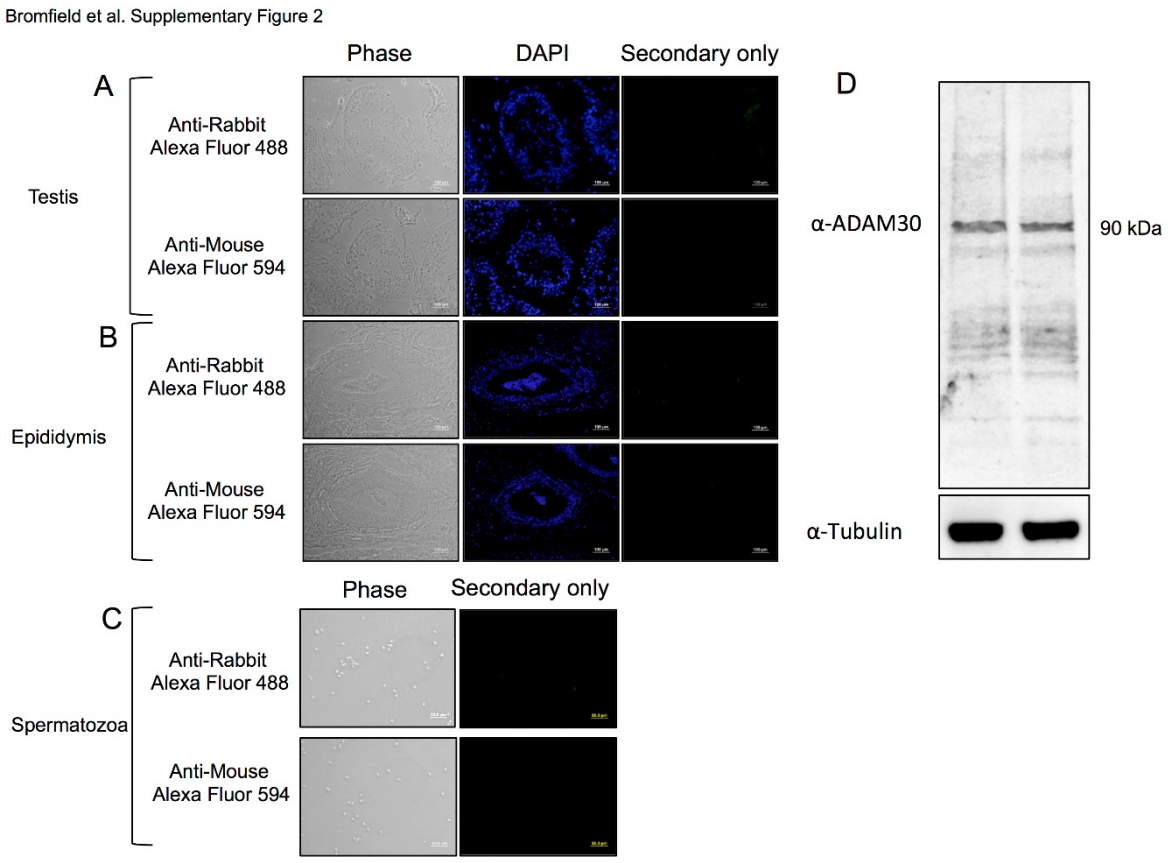
Chapter 3: Supplementary material



Supplementary Figure 1. Analysis of human testis and epididymis tissue morphology. (A) Tissue morphology was assessed using haemotoxylin/eosin staining, revealing relatively poor preservation of the structural integrity of the testis samples. Despite this, some tubules did contain mature germ cells and these tubules formed the focus of our analyses of the expression of BAG6 and HSPA2 as presented in Figure 2. (B) Haemotoxylin/eosin staining of epididymal tissue sections revealed a relatively high concentration of luminal spermatozoa in the proximal region of the duct and, with the exception of some abnormal vacuolisation, the tissue morphology appeared otherwise normal. Images were captured on a Zeiss axiovert fluorescence microscope using phase/contrast settings. Scale is indicated on each image.



Supplementary Figure 2. Secondary alone controls for testis, epididymis and isolated spermatozoa. (A-B) Confocal microscopy was performed on testis and epididymis tissue sections incubated with either anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 594 in absence of primary antibodies and counterstained with DAPI. Images were captured using a 40× objective. (C) Similarly, confocal images of isolated spermatozoa were subjected to the same 'secondary alone' treatments and fluorescence and phase-contrast images were captured using a 40× objective. Scale is indicated on each image. (D) To confirm the presence of ADAM30 in non-capacitated and capacitated spermatozoa, sperm lysates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes. These membranes were incubated with anti-ADAM30 primary antibodies and corresponding secondary antibodies and cross-reactive proteins were visualized using enhanced chemiluminescence. Membranes were re-probed with anti-tubulin to determine loading equivalency.

CHAPTER 4:

HSPA2 forms a stable complex with Angiotensin Converting Enzyme (ACE) and Protein Disulfide Isomerase A6 (PDIA6) in human spermatozoa

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Chapter 4: Overview

In the studies documented in the previous chapters we have confirmed that the attenuation of HSPA2 chaperone activity has deleterious effects on ZP-receptor complex function in human spermatozoa and that BAG6 may be an important regulator of HSPA2 stability in human germ cells. Given these findings, we were interested in determining additional client-proteins of HSPA2 that may be affected by its deficiency in the patient population. This would allow us to further understand the pathology of this condition as well as determine the extent to which HSPA2 governs sperm function. In this light, the studies described in this chapter aimed to identify and characterize additional interacting-partners of HSPA2 in human spermatozoa.

Through this work we have identified Angiotensin Converting Enzyme (ACE) and Protein Disulfide Isomerase A6 (PDIA6) as potential HSPA2-client proteins and have demonstrated that this protein complex resides in membrane raft microdomains located in the peri-acrosomal region of the sperm head. Additionally, the surface expression of PDIA6, was shown to be dynamically regulated during sperm capacitation. Like that of the previously characterised HSPA2 client proteins, SPAM1 and ARSA, this translocation also proved vulnerable to oxidative stress. Moreover, pharmacological inhibition of ACE significantly reduced the ability of human spermatozoa to undergo an agonist induced acrosome reaction suggesting that this protein complex is functionally significant in human spermatozoa.

In view of these findings, we propose that ACE and PDIA6 are novel client-proteins of the chaperone HSPA2 and that this complex may participate in key elements of the fertilization cascade. As HSPA2 is compromised in the spermatozoa of men with oocyte recognition defects, the characterization of these HSPA2-client proteins provides important insight into the complexity of the cellular pathways that may be affected in the spermatozoa of infertile individuals.

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HSPA2 forms a stable complex with Angiotensin Converting Enzyme (ACE) and Protein Disulfide Isomerase A6 (PDIA6) in human spermatozoa

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Key Words:	acrosome reaction, angiotensin, infertility, zona pellucida, spermatozoa

ABSTRACT

Study hypothesis: Given the importance of the chaperone Heat Shock Protein A2 (HSPA2) in the regulation of male fertility, this study aimed to identify and characterize additional proteins that may rely on the activity of this chaperone in human spermatozoa.

Study finding: In view of the findings in this study we propose that Angiotensin Converting Enzyme (ACE) and Protein Disulfide Isomerase A6 (PDIA6) are novel interacting proteins of HSPA2 and that this multimeric complex may participate in key elements of the fertilization cascade.

What is known already: The molecular chaperone HSPA2 plays a pivotal role in the remodelling of the sperm surface during capacitation. Indeed, human spermatozoa that are deficient in HSPA2 protein expression lack the ability to recognize human oocytes, resulting in repeated IVF failure in a clinical setting. Moreover, our recent work has shown that defective HSPA2 function induced by oxidative stress leads to the aberrant surface expression of one of its interacting proteins, arylsulfatase A, and thus contributes to a loss of sperm-ZP adhesion.

Study design, samples/materials, methods: Human spermatozoa were collected from fertile donors, capacitated and prepared for Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) analysis. Protein complexes resolved via BN-PAGE were excised and their constituents were identified using mass spectrometry. The interactions between ACE, PDIA6 and HSPA2 were then confirmed using immunoprecipitation and proximity ligation assays and the localisation of these proteins was assessed in isolated spermatozoa and commercially available human testis tissue sections. Finally, pharmacological inhibition of ACE was performed to assess the role of ACE in human sperm capacitation.

Main results and the role of chance: Herein we have identified ACE and PDIA6 as potential HSPA2-interacting proteins and shown that this assemblage resides in membrane raft microdomains located in the peri-acrosomal region of the sperm head. Additionally, the surface expression of PDIA6, but not ACE, was shown to be dynamically regulated during sperm capacitation and, like that of previously characterized HSPA2-interacting proteins, this surface expression proved vulnerable to

oxidative stress. In terms of the functional significance of this protein complex, pharmacological inhibition of ACE significantly reduced the ability of human spermatozoa to undergo an agonist induced acrosome reaction ($P<0.01$).

Limitations, reasons for caution: While these results provide a descriptive analysis of the PDIA6/ACE/HSPA2 complex, this study provides the impetus for further investigation into the role of PDIA6 and ACE in human sperm function.

Wider implications of the findings: As our research group, and others, have shown that HSPA2 is compromised in the spermatozoa of men with oocyte recognition defects, the characterization of these HSPA2-interacting proteins provides important insight into the complexity of the cellular pathways that may be affected in the spermatozoa of infertile individuals.

Large scale data: Large scale proteomics data can be accessed through the Proteomics Identifications Database (PRIDE).

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Key Words: acrosome reaction, angiotensin, infertility, zona pellucida, spermatozoa

INTRODUCTION

Notwithstanding a transient period of interaction with the epithelial cells of the oviduct, spermatozoa possess the unique ability to disregard the presence of the millions of cells they encounter within the female reproductive tract before undergoing a resolute interaction with the ovulated oocyte. Given this, it is no surprise that spermatozoa possess a highly sophisticated cell receptor system for recognition of the outer vestments of the egg, the zona pellucida (ZP; Asquith et al., 2004; Dun et al., 2011; Bernabo et al., 2014; Tanphaichitr et al., 2015). Nevertheless, efforts to tease out the importance of molecules with affinity for the ZP using gene manipulation technology have revealed a high level of redundancy in receptor protein function (As reviewed by Okabe et al., 2015), perhaps to ensure the soundness of the important interface that leads to fertilization.

Our previous work has shown that the collaborative action of sperm proteins at the cell surface is reliant upon the activity of molecular chaperones to ensure the correct temporal and spatial location of ZP receptors (Dun et al., 2011; Redgrove et al., 2011; 2012; 2013). In the case of human spermatozoa, the assembly of a majority of these protein complexes appears to be regulated by the chaperoning activity of Heat Shock Protein A2 (HSPA2; Redgrove *et al.,* 2012; Bromfield et al., 2015a). While the function of this chaperone is known to be critical throughout spermatogenesis (Huszar et al., 1997; as reviewed by Scieglinska and Krawczyk, 2015), its implication in protein complex regulation in mature spermatozoa has recently been examined by exploring the dynamics of two of its ZP receptor-substrates: Sperm Adhesion Molecule 1 (SPAM1) and Arylsulfatase A (ARSA) (Redgrove et al., 2012).

During the processes of epididymal maturation and capacitation, the sperm surface experiences a dynamic transformation to ensure the correct suite of proteins reach the sperm surface in preparation for ZP adhesion (Phillips et al., 1991; Gadella et al., 2008; Tsai et al., 2010). A key example of this process lies in the HSPA2-dependent reorganization of SPAM1

and ARSA that culminates in the timely presentation of ARSA at the surface of capacitated spermatozoa prior to ZP interaction (Redgrove et al., 2012; 2013). However, this tightly regulated event can be disrupted by the accumulation of oxidative stress whereupon the affected spermatozoa have severely reduced ARSA surface expression and a limited ability to interact with human ZP (Bromfield et al., 2015b). Furthermore, this defect is tied to the modification and subsequent attenuation of HSPA2 function through chemical modification of the chaperone by the lipid peroxidation product 4-hydroxynonenol (4HNE). While model systems have taught us that the functions of numerous HSP70 family proteins are interchangeable (Kabani and Martineau, 2008), it seems that in spermatozoa the biological function that is lost with this particular isoform (HSPA2) does not appear to be supported by the action of other HSP70 proteins present in the cell (Redgrove et al., 2012; Bromfield et al., 2015b). This is particularly alarming as a cohort of infertile patients presenting with sperm-egg recognition defects, the cause of repeated *in vitro* fertilization (IVF) failure, possess spermatozoa that are deficient in HSPA2 protein expression (Ergur et al., 2002; Redgrove et al., 2012; Motiei et al., 2013).

As we have confirmed that the attenuation of HSPA2 function has deleterious effects on protein complex function in human spermatozoa (Bromfield et al., 2015b), we were interested in determining additional binding-proteins of HSPA2 that may be affected by its deficiency in the patient population. This would allow us to further understand the pathology of this condition as well as determine the extent to which HSPA2 governs sperm function. In this light, the current study aimed to identify and characterize additional interacting-partners of HSPA2 in human spermatozoa. Specifically, we provide evidence for the novel association of HSPA2 with the testicular isoform of angiotensin converting enzyme (tACE), the ablation of which results in severe subfertility in gene-manipulated mice (Kessler et al., 2000), and protein disulfide isomerase A6, (PDIA6; previously ERP5), an important candidate for the

regulation of cell-cell adhesion in biological systems, and discuss the putative roles of these proteins in relation to human sperm function.

MATERIALS AND METHODS

Ethical Approval

The experiments described in this study were conducted using human semen samples obtained from a panel of healthy normozoospermic donors in accordance with the University of Newcastle's Human Ethics Committee guidelines (Approval No. H-2013-0134).

Reagents

Unless specified, research grade chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were purchased to characterize proteins of interest: a mouse monoclonal antibody to angiotensin converting enzyme (ACE) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) [Cat # Sc-23908]), a rabbit polyclonal anti-protein disulfide isomerase 6 (PDIA6) was purchased from Sigma (Cat # HPA034653) and a rabbit polyclonal antibody to HSPA2 was from Sigma-Aldrich (Cat # HPA000798). Albumin and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Research Organics (Cleveland, OH, USA). D-glucose, sodium hydrogen carbonate, sodium chloride, potassium chloride, calcium chloride, potassium orthophosphate, and magnesium sulfate were all analytical reagent grade, purchased from Merck (BDH Merck, Kilsyth, VIC, Australia). Tris was from ICN Biochemicals (Castle Hill, NSW, Australia), and Percoll from (Rydalmere, NSW, Australia). Nitrocellulose was from GE Healthcare (Buckinghamshire, UK) while highly pure Coomassie brilliant blue G250 was obtained from Serva (Heidelberg, Germany). SYTOX green cell vitality stain was purchased

from Invitrogen (Carlsbad, CA, USA). Mowiol 4-88 was from Calbiochem (La Jolla, CA, USA), and paraformaldehyde was supplied by ProSciTech (Thuringowa, Australia). Appropriate HRP conjugated secondary antibodies were obtained from Santa Cruz Biotechnology and Sigma-Aldrich. Testis sections were purchased from Abcam (Cat # Ab4373) and proximity Ligation Assay reagents were purchased from Duolink (OLINK Biosciences, Uppsala, Sweden).

Human sperm preparation

Enrichment of human spermatozoa was achieved using 45% and 90% discontinuous Percoll gradients as described previously (Bromfield et al., 2013). High quality spermatozoa were recovered from the base of the 90% Percoll fraction and resuspended in a bicarbonate-free non-capacitating (NC) form of Biggers, Whitten and Whittingham medium (NC BWW; Biggers et al. 1979) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin and 20 mM HEPES buffer and 1 mg/ml polyvinyl alcohol (osmolarity of 300 mOsm/kg). The cells were then pelleted by centrifugation at 500 × *g* for a further 15 min and resuspended at a concentration of 10×10^6 cells/ml.

Sperm capacitation

To induce capacitation *in vitro*, human spermatozoa were resuspended in a capacitating (CAP) form of BWW composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin, 20 mM HEPES

buffer and 1 mg/ml polyvinyl alcohol (PVA) (osmolarity of 300mOsm/kg) and supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic adenosine monophosphate (dbcAMP). Cells were incubated in this medium for 3 h at 37°C under an atmosphere of 5% CO_2: 95% air with cells resuspended at a concentration of 10×10^6 cells/ml. Non-capacitated cells were incubated for the same period of time (without exposure to CO_2) in BWW prepared without $NaHCO_3$ (NC BWW). Following incubation, cell motility was assessed through phase-contrast microscopy. Populations of spermatozoa were then used for the assays outlined below.

Blue Native polyacrylamide gel electrophoresis (BN-PAGE)

Capacitated spermatozoa were prepared for BN-PAGE as previously described (Redgrove *et al*., 2013). Briefly, cell pellets were resuspended in native lysis buffer consisting of 1% n-dodecyl b-D-maltoside, 0.5% Coomassie Blue G250 and a cocktail of protease inhibitors (Roche, Mannheim, Germany) and incubated at 4°C on an orbital rocker for 30 min. Samples were centrifuged for 20 min at 14 000 × g and then dialyzed against Blue Native cathode buffer (purchased from Invitrogen, Carlsbad, CA, USA) overnight at 4°C. Dialyzed native protein lysates were loaded onto blue native polyacrylamide gels (NativePAGE Novex 4-16%, Bis-Tris gels; Invitrogen) and resolved using a NativePAGE cathode and anode buffer (Redgrove *et al*., 2011) and run at 4°C at 100V for the duration of the stacking gel layer and at 200V for the resolving gel layer for ~3 h. Following retrieval from the cassettes, gels were either stained with Coomassie G250 or prepared for western blotting.

Mass Spectrometry and protein identification

Mass spectrometry (MS) analyses were performed at the Australian Proteome Analysis Facility using a one-dimensional (1D) nano-liquid chromatography electrospray ionization

MS/MS interface, as previously described (Redgrove *et al*., 2011). Peptide data were exported in a format suitable for submission to the database search program, Mascot (Matrix Science Ltd, London, UK). Peaklists were searched against Homo sapiens in the SwissProt database (2013). High scores in the database search indicate a likely match, which was confirmed or qualified by operator inspection. Search results were generated with a significance threshold of $P < 0.02$ with a cut-off score of 34 for all samples and a peptide mass tolerance of +300 ppm, a fragment mass tolerance of +0.6 Da, and maximum trypsin missed cleavages set to 1.

SDS-PAGE and Western blotting

Following treatment, human spermatozoa were pelleted via centrifugation and resuspended for protein extraction as previously described (Reid *et al*., 2012). Protein extracts were then boiled in the presence of NuPAGE LDS sample buffer (Invitrogen) containing 8% β-Mercaptoethanol, subjected to SDS-PAGE using 4-12% BIS-TRIS gels (Invitrogen) and then electro-transferred to nitrocellulose membranes using conventional western blotting techniques (Towbin *et al*., 1979). To detect proteins of interest, membranes were blocked in 3% BSA in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST, pH 7.4) then probed with either anti-HSPA2 (diluted 1:1000), anti-ACE (1:500), anti-PDIA6 (1:1000) or anti-tubulin (1:4000) in TBST supplemented with 1% BSA under constant rotation overnight at 4°C. Membranes were washed in TBST (3 × 10 min) and labelled proteins were visualized using an enhanced chemiluminescence detection kit (ECL plus, GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

Immunohistochemistry

Embedded testis tissue was dewaxed and rehydrated as previously described (Reid et al., 2012) and sections were subjected to antigen retrieval via immersion in 10 mM sodium citrate

(pH 6) and microwaving at 1000 W for 10 min. Subsequent incubations were performed as previously described by Reid et al. (2012) with primary antibodies diluted 1:50 (ACE; PDIA6) or 1:100 (HSPA2) and incubated overnight at 4°C. Following incubation in appropriate Alexa Fluor conjungated secondary antibodies (1:200), the slides were washed and mounted using a MOWIOL anti-fade reagent (13% Mowiol 4-88, 33% glycerol, 66 mM Tris, pH 8.5, 2.5% 1,4-diasabicyclo-[2,2,2] octane) and viewed with an Avio Imager A1 fluorescence microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA) with images taken on an Olympus DP70 microscope camera.

Immunocytochemistry

Following capacitation, spermatozoa were fixed in 4% paraformaldehyde, washed three times with 0.05M glycine in phosphate-buffered saline (PBS) and then applied to poly-L-lysine coated glass coverslips. Cells were permeabilized with 0.2% Triton X-100, then placed in a humid chamber and blocked in 3% BSA/PBS for 1 h. Coverslips were then washed in PBS and incubated in appropriate primary antibodies diluted 1:100 (anti-HSPA2), 1:50 (anti-ACE; anti-PDIA6) or 1:100 (anti-phosphotyrosine PT66) with 1% BSA/PBS overnight at 4°C. Following this, coverslips were washed (3 × 5 min) in PBS before applying appropriate secondary antibodies diluted 1:100 with 1% BSA/PBS for 1 h at RT. Coverslips were washed in PBS (3 × 5 min) before mounting in 10% mowiol 4-88 (Calbiochem) with 30% glycerol in 0.2M Tris (pH 8.5) and 2.5% 1,4-diazobicyclo-(2.2.2)-octane (DABCO). Immunolocalization of target antigens were examined with a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss Pty, Sydney, Australia). Human spermatozoa were classified as phosphotyrosine (PT66) positive when displaying uniform fluorescence across the full length of the sperm flagella, as previously described (Urner *et al*., 2003).

Duolink Proximity Ligation Assay

Duolink in situ primary ligation assays (PLAs) were conducted in accordance with the manufacturers' instructions on fixed human spermatozoa adhered to poly-L-lysine coated coverslips (OLINK Biosciences, Uppsala, Sweden). Briefly, samples were blocked in Duolink blocking solution and then incubated with primary antibodies (anti-ACE, anti-PDIA6, anti-HSPA2, and anti-Tubulin) overnight at 4°C. Oligonucleotide conjugated secondary antibodies (PLA probes) were then applied for 1 h at 37°C and ligation of the PLA probes was performed and the signal amplified according to the manufacturer's instructions. The fluorescent signal generated when molecules are in close association (< 40 nm) was visualized using fluorescence microscopy and could be quantified by recording the proportion of 100 spermatozoa displaying a collection of red fluorescent dots over the sperm head. Specificity of this reaction was ensured by performing proximity ligation with antibodies to the target antigens combined with anti-tubulin antibodies with which they should not interact.

Immunoprecipitation

Non-capacitated and capacitated spermatozoa were prepared as above and cell lysis was performed on populations of ~100 × 10^6 cells from each treatment at 4°C for 2 h in lysis buffer consisting of 10mM CHAPS, 10mM HEPES, 137 mM NaCl and 10% glycerol with the addition of protease inhibitors (Roche). The cell lysates were then added to 50 µl aliquots of washed protein G Dynabeads and incubated under rotation to preclear at 4°C for 1 h. Anti-HSPA2 antibody, 10 µg in 200µl of PBS, was conjugated to fresh aliquots of washed (supernatant removed) Dynabeads by incubation for 2 h at 4°C under rotation. Following antibody binding, the cross-linking reagent, 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP), was added at a final concentration of 2 mM and crosslinking was performed at RT

for 30 minutes after which 20 mM TRIS was added to each tube for an additional 15 minutes at RT to quench the reaction.

Immunoprecipitation was then performed by adding 1 ml pre-cleared lysate to HSPA2 antibody bound beads and incubating under rotation overnight at 4°C. After incubation, supernatants were transferred to clean tubes and washed (3×) in 200 µl of PBS. Target antigen was eluted from the beads by boiling in the presence of SDS loading buffer containing 8% β-Mercaptoethanol. The same elution step was performed on preclear beads and these solutions were loaded onto a NuSep 4-20% TRIS-Glycine gel for analysis via SDS-PAGE. In addition, bead only and antibody only controls were prepared by loading 10 µl of protein-G bead slurry and 5 µl of anti-HSPA2 in the presence of SDS-loading buffer into appropriate gel lanes. Gels were loaded in triplicate, resolved at 150V for ~1 h and prepared for immunoblotting with anti-ACE, anti-HSPA2 and anti-PDIA6 antibodies.

Inhibition of Angiotensin converting enzyme

Captopril, an extensively studied inhibitor of ACE was utilized at doses that have been previously recognized to be effective for the inhibition of ACE in human spermatozoa (Foresta et al., 1991). Human spermatozoa were incubated in capacitating media supplemented with 25, 50, 100, 150 and 200 µM captopril (Cat # C4042; Sigma) throughout the 3 h capacitation period. Following this incubation, spermatozoa were washed with BWW and prepared as specified below.

Membrane fluidity and membrane raft labelling

Merocyanine 540 (Sigma) was used to assess membrane fluidity of human spermatozoa. Following capacitation, aliquots of human sperm from each treatment were diluted to 1×10^6 cells/ml and incubated in SYTOX green vitality stain at 37°C for 10 min. This preparation

was washed once in BWW and then incubated in 2.7 μM merocyanine 540 at 37°C for 10 min. Preparations were then washed once in BWW and 200 cells from each treatment were scored on a Zeiss fluorescence microscope at excitation wavelengths 590 nm (merocyanine 540), 470 nm (SYTOX green) (Carl Zeiss, Thornwood, NY, USA). Merocyanine positive sperm were identified through bright red fluorescence over the entire head and the absence of SYTOX green staining. The localization of membrane raft marker, G_{M1} ganglioside, was visualized in human spermatozoa by staining with Alexa Fluor 488-labelled B subunit of cholera toxin (CTB) as previously described (Nixon *et al.*, 2011). For each treatment, 200 cells were classified into two fluorescent patterns (head and tail or head only labelling) and representative images were taken.

Acrosome reaction

To induce acrosomal exocytosis, human spermatozoa were incubated for 30 min with 1.25 μM calcium ionophore (A23187). Sperm were then incubated in pre-warmed hypo-osmotic swelling media (HOS; 0.07% w/v sodium citrate; 1.3% w/v fructose) for a further 30 min at 37°C. Sperm preparations were washed in PBS, placed on poly-L-lysine coated slides and allowed to air dry. The cells were then permeabilized in ice-cold methanol and subjected to immunocytochemistry with tetramethylrhodamine (TRITC) labelled peanut agglutinin lectin (PNA, 1:200), as previously described (Redgrove *et al*., 2013). The acrosomal status of human spermatozoa was assessed with a Zeiss LSM510 laser scanning confocal microscope. Acrosome reacted cells were identified by the appearance of a curled tail and either the complete absence of PNA staining over the acrosomal region or the restriction of this labelling to the equatorial segment of the sperm head.

Induction of oxidative stress in Human Spermatozoa

Oxidative stress was induced in populations of non-capacitated human spermatozoa through treatment with 50 μM hydrogen peroxide (H_2O_2) as described previously (Bromfield et al., 2015b). Cells at a concentration of 10×10^6 cells/ml were resuspended in H_2O_2 and then incubated for 1 h at 37°C. Treated spermatozoa were then washed once in non-capacitating BWW (NC) and then capacitated under standard capacitating conditions or in the presence of capacitating media supplemented with 1mM D-penicillamine (as described by Bromfield et al., 2015b).

Surface labelling of human sperm proteins

Non-capacitated, capacitated, H_2O_2 treated and penicillamine treated sperm suspensions were incubated with primary antibodies, Anti-ACE (1:50), Anti-PDIA6 (1:50), Anti-HSPA2 (1:100) or Anti-CD59 (1:100) for 1 h. The cells were subsequently washed 2 × with BWW and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (diluted 1:200) for a further 30 min. Following three washes with BWW, the cells were incubated with 20 mg/ml of propidium iodide (PI) for 1 min and assessed for surface fluorescence using a Zeiss fluorescence microscope. Anti-CD59 was used as a positive control for surface expression in human spermatozoa and the proportion of sperm expressing ACE, PDIA6 or HSPA2 on their surface was recorded for 200 cells from each treatment.

Statistical analyses

All experiments were replicated at least 3 × with independent samples and data are expressed as mean values ± S.E. Statistical analysis was performed using a two-tailed, unpaired Student's t test using Microsoft Excel (Version 14.0.0). Differences were considered significant for $p < 0.05$.

RESULTS

Identification of HSPA2-interacting proteins and confirmation of their interaction through BN-PAGE and immunoprecipitation

To identify potential binding proteins of the chaperone HSPA2 in capacitated human spermatozoa, liquid chromatography mass spectrometry (LC-MS) was performed on protein bands excised from BN-PAGE gels. BN-PAGE gels were aligned with anti-HSPA2 Western blots (As shown in Figure 1A) and two corresponding HSPA2-laden complexes were prepared for MS analysis (as previously described by Redgrove et al., 2013).

The identification of peptides corresponding to HSPA2 was confirmed for each excised band and from these analyses, two key complexes of ~280 kDa and ~180 kDa, respectively, were chosen for characterization. In addition to HSPA2, the larger of these complexes was found to contain a metalloprotease of the ADAM family of proteins (ADAM30) and BCL-2 associated athanogene 6 (BAG6), a known regulator of HSPA2 stability (Sasaki et al., 2008). The characterization of this complex throughout human germ cell development was recently reported (Bromfield et al., 2015a).

Within the smaller ~180 kDa complex, high confidence identifications were returned for seven peptides uniquely corresponding to Angiotensin Converting Enzyme (ACE), a single peptide mapping to Protein Disulfide Isomerase A6 (PDIA6), and 10 peptides uniquely corresponding to human HSPA2 (Table I). Given the well-characterized role of ACE and the protein disulfide isomerase (PDI) family in mouse sperm-egg interaction, the current study focused on investigating the interaction between these proteins in human spermatozoa.

Based on previous studies it is known that human tissues harbor two isoforms of ACE. One of these is a somatic isoform of ACE (sACE, ~140-180 kDa) that has been implicated in the renin-angiotensin system (Skeggs, 1993) and the second is a germinal or testicular form of

ACE that is expressed exclusively in post-meiotic male germ cells (tACE; ~80-110 kDa) (El-Dorry et al., 1982; Howard et al., 1990). As the peptides identified in our mass spectrometry analysis did not map exclusively to tACE, a monoclonal anti-ACE antibody (2E2) that detects both isoforms was used throughout this study.

Table I: Identification of potential HSPA2-client proteins, Angiotensin Converting Enzyme (ACE) and Protein Disulfide Isomerase A6 (PDIA6), in a human sperm protein complex.

Protein (symbol)	UniProt Accession number	Number of unique peptides matched	Unique peptide sequences	Mascot score for individual peptides	Overall mascot score
Heat shock 70kDa protein 2 (HSPA2)	P54652	10	K.DAGTITGLNVLR.I K.MKEIAEAYLGGK.V K.NALESYTYNIK.Q K.CQEVINWLDR.N K.GQIQEIVLVGGSTR.I K.TFFPEEISSMVLTK.M K.NQVAMNPTNTIFDAK.R K.LDKGQIQEIVLVGGSTR.I K.VHSAVITVPAYFNDSQR.Q K.LYQGGPGGGSGGGGSGASGGPTIEEVD.-	112 55 78 78 107 102 127 68 90 81	1068
Angiotensin Converting Enzyme (ACE)	P12821	7	R.AILQFYPK.Y K.YQGLCPPVPR.T + Carbamidomethyl (C) R.AALPAQELEEYNK.I K.ENYNQEWWSLR.L R.VSFLGLDLDAQQAR.V K.LGWPQYNWTPNSAR.S R.SMYETPSLEQDLER.L R.SMYETPSLEQDLER.L + Oxidation (M)	54 39 37 89 49 61 96 (85)	280
Protein Disulfide Isomerase A6 (PDIA6)	Q15084	1	R.TGEAIVDAALSALR.Q	91	91

The presence of each protein constituent of the putative complex was first verified in non-capacitated and capacitated human sperm lysates through immunoblotting (Figure 1B-D). Probing these blots with appropriate primary antibodies confirmed the expected presence of a ~70 kDa band corresponding to HSPA2 (Figure 1A), both sACE and tACE isoforms (~140 kDa and ~80 kDa, respectively) (Figure 1B), and a single band at ~48 kDa corresponding to the PDIA6 (Figure 1C) in populations of both non-capacitated and capacitated human spermatozoa.

To evaluate the interaction between these proteins, BN-PAGE was used to resolve native protein complexes from capacitated human spermatozoa (Figure 1D). Labelling of BN-PAGE immunoblots with anti-HSPA2, anti-ACE and anti-PDIA6 antibodies revealed that these three proteins partitioned into a predominant complex with an aggregate molecular weight of ~180 kDa. This approximately equates to the combined molecular weights of the HSPA2, tACE, and PDIA6 proteins, thus suggesting they are capable of forming a stable complex under these experimental conditions (Figure 1D). As we have documented for HSPA2 (Redgrove et al., 2012), the labelling of each protein was not restricted to this single complex. Indeed, anti-ACE antibodies also labelled two lower molecular weight bands of ~140 and 80 kDa which likely correspond to the sACE and tACE monomers. Similarly, anti-PDIA6 also cross-reacted with an additional four complexes of aggregate molecular weights exceeding 240 kDa, however a band corresponding to the monomeric form of PDIA6 protein was not detected (Figure 1D).

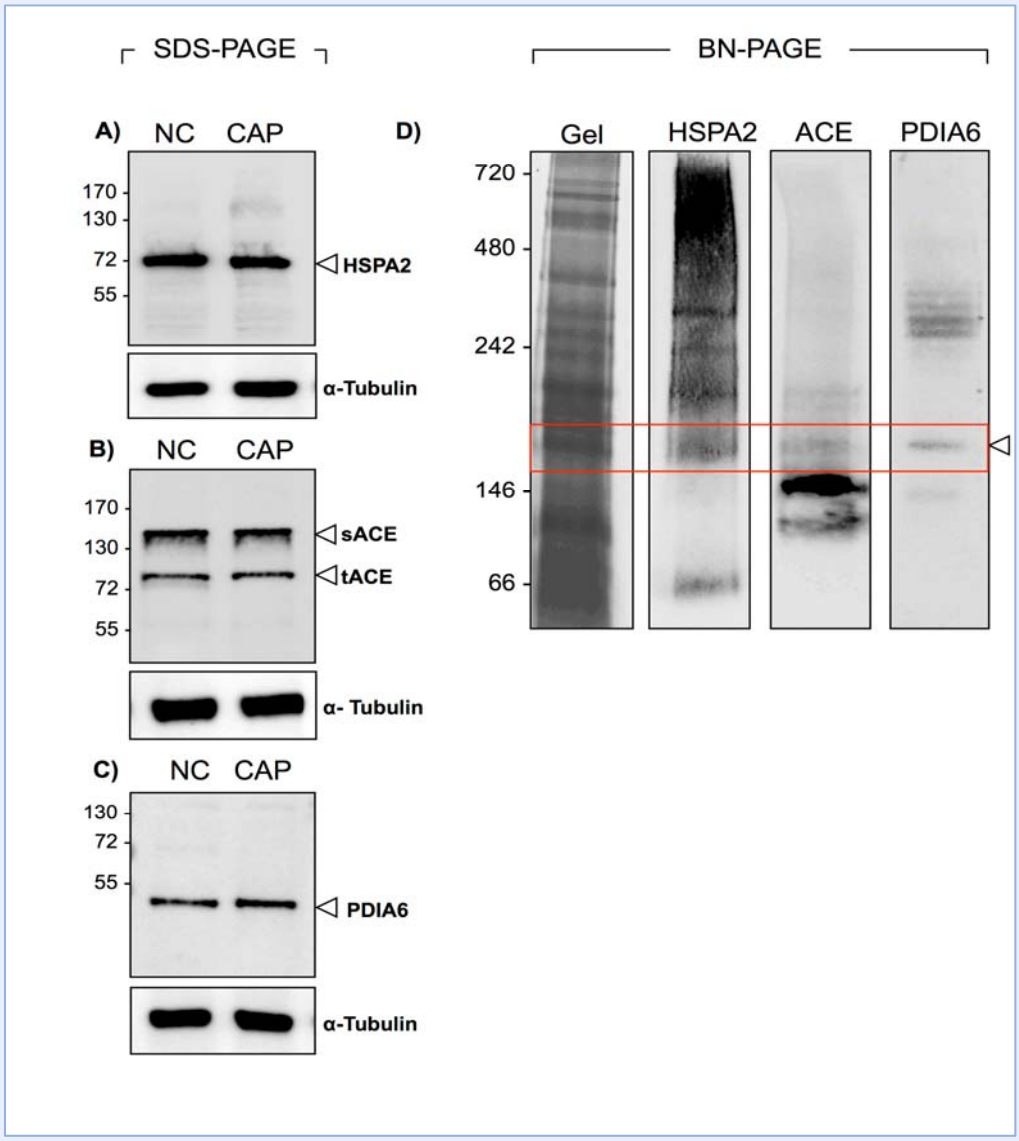

Figure 1: Confirmation of ACE and PDIA6 expression in human spermatozoa through Immunoblotting analyses of proteins resolved via SDS-PAGE and BN-PAGE. (A-C) The presence of Heat Shock Protein A2 (HSPA2), Angiotensin Converting Enzyme (ACE) and Protein disulfide Isomerase A6 (PDIA6) was confirmed in both non-capacitated and capacitated SDS-extracted lysates using conventional Western blotting techniques. D) Native lysates from capacitated spermatozoa were resolved using Blue Native-PAGE and protein complexes were visualized by Coomassie staining or prepared for Western blotting. Probing BN-PAGE blots with anti-HSPA2 resulted in the detection of this protein in five key protein complexes of aggregate molecular weights >100 kDa. Probing of corresponding blots with anti-ACE revealed the presence of its monomeric forms at ~150 kDa (sACE) and ~90 kDa (tACE) and its presence in a dominant complex with an aggregate molecular weight of ~180 kDa (denoted by an arrowhead) that co-migrated with HSPA2. Probing an equivalent immunoblot with anti-PDIA6 confirmed the presence of this protein within the same complex and also revealed an additional subset of high molecular weight complexes (270-320 kDa) labeled with PDIA6.

To further confirm the interaction between HSPA2, ACE and PDIA6, non-capacitated and capacitated sperm lysates were precipitated with anti-HSPA2 and the eluates were tested for the presence of each target protein. As shown in Figure 2A, this technique was effective in isolating the bait protein, HSPA2, along with its corresponding targets, ACE and PDIA6. As shown in Figure 2B, dominant bands of ~140 kDa and ~80 kDa corresponding to sACE and tACE, respectively, were present in both the lysate and elution lanes for non-capacitated and capacitated spermatozoa. Similarly in Figure 2C, bands of ~48 kDa were detected in the lysate controls and elution lanes upon probing for anti-PDIA6. The specificity of the immunoprecipitation assay was verified through inclusion of antibody-only (Ab-only) and bead-only controls in addition to the preclear bead eluate (Figure 2A-C).

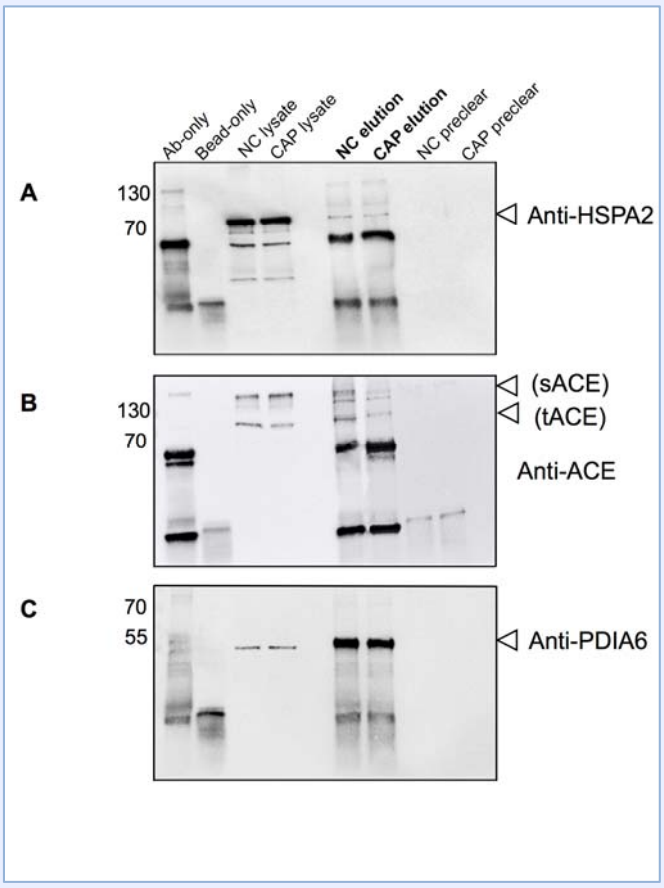

Figure 2: Confirmation of the interaction of HSPA2-client proteins through immunoprecipitation. (A-C) Confirmation of interactions between HSPA2-client proteins in non-capacitated and capacitated spermatozoa was sought using an immunoprecipitation approach in which HSPA2 was used as bait to pull down interacting partners. The captured sperm proteins were eluted from protein G beads and resolved on SDS-PAGE gels alongside an antibody-only control (Ab control), a bead-only control (Bead control), non-capacitated (NC) and capacitated (CAP) sperm lysate and precleared controls. (A) HSPA2 IP blots were probed with anti-HSPA2, and the specificity of the IP was confirmed through the detection of a 72 kDa band in both the elution and lysate lanes but importantly not in the control lanes. Probing corresponding blots with anti-ACE (B) revealed the presence of sACE and tACE in both the IP eluates at 80 kDa and 140 kDa, respectively, and in the lysate lanes (denoted by an arrowhead) but again not in the control lanes. (C) Probing corresponding blot with anti-PDIA6 resulted in the detection of PDIA6 at ~50 kDa in both the eluates and in the lysate lanes but not in the control lanes. This assay indicated that both ACE and PDIA6 could be immunoprecipitated from non-capacitated and capacitated human spermatozoa using anti-HSPA2.

Localization of ACE, PDIA6 and HSPA2 in the human testis

Given that a putative interaction between ACE, PDIA6 and HSPA2 was supported by our BN-PAGE and immunoprecipitation results, we next sought to investigate the whether this interaction originates during germ cell development in the human testis. Although the ability to study protein interactions in testicular germ cells is compromised by limited access to non-pathological human testis material, testis sections were sourced from a commercial supplier and the localization of ACE, PDIA6 and HSPA2 was explored using immunohistochemical analysis. Notwithstanding the poor morphology within these sections that restricted our ability to identify precise stages of germ cell development, we were nonetheless able to demonstrate that the HSPA2 chaperone was present in both early and later stage germ cells (Figure A, H). In contrast, ACE labelling was restricted to later stage (likely post-meiotic) germ cells (Figure 3B, D) with clear co-localization between HSPA2 and ACE observed in these cells (Figure 3C). The presence of PDIA6 was observed in both early and later stage germ cells (Figure 3E, G) with additional labelling of the peripheral spermatogonia. HSPA2 was also detected in these peripheral cells of the testis and co-localization of HSPA2 and PDIA6 was clearly seen in this region (Figure 3I) in addition to that of later stage germ cells. In contrast, co-localization of ACE and PDIA6 was revealed solely in later stage germ cells (Figure 3F). In view of the co-localization of PDIA6, ACE and HSPA2 in the maturing germ cells of the testis we next sought to confirm the interaction between these proteins in mature spermatozoa.

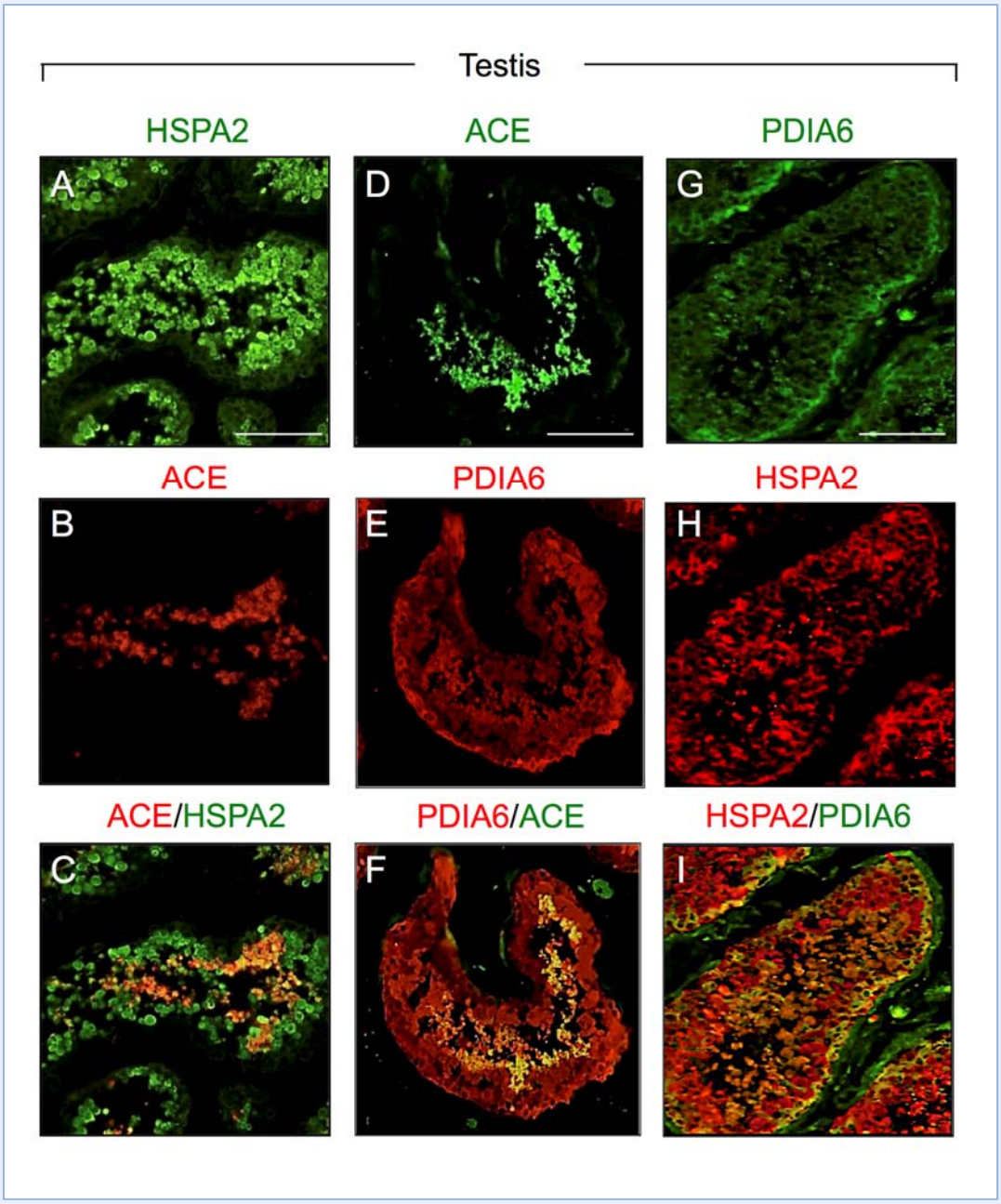

Figure 3: Detection of HSPA2, ACE and PDIA6 in the human testis. (A-I) Immunohistochemistry was performed on human testis tissue using antibodies against each protein and fluorescence images were taken on a × 40 objective. (C) Dual labeling was performed with anti-ACE/anti-HSPA2, (F) anti-PDIA6/anti-ACE and (I) anti-HSPA2/anti-PDIA6. Colocalization was most distinct in the later stage germ cells (spermatocytes, developing spermatids and spermatozoa) with additional co-localization observed in the peripheral spermatogonia when labeled with anti-PDIA6/anti-HSPA2 (I). Scale bars = 100 µm.

Localization of ACE, PDAI6 and HSPA2 in non-capacitated and capacitated spermatozoa

The localization of HSPA2, ACE, and PDIA6 in human spermatozoa was investigated through immunocytochemistry, revealing that each protein clearly resided within the peri-acrosomal region of the sperm head (Figure 4A) in over 80% of both non-capacitated and capacitated sperm populations (Figure 4C).

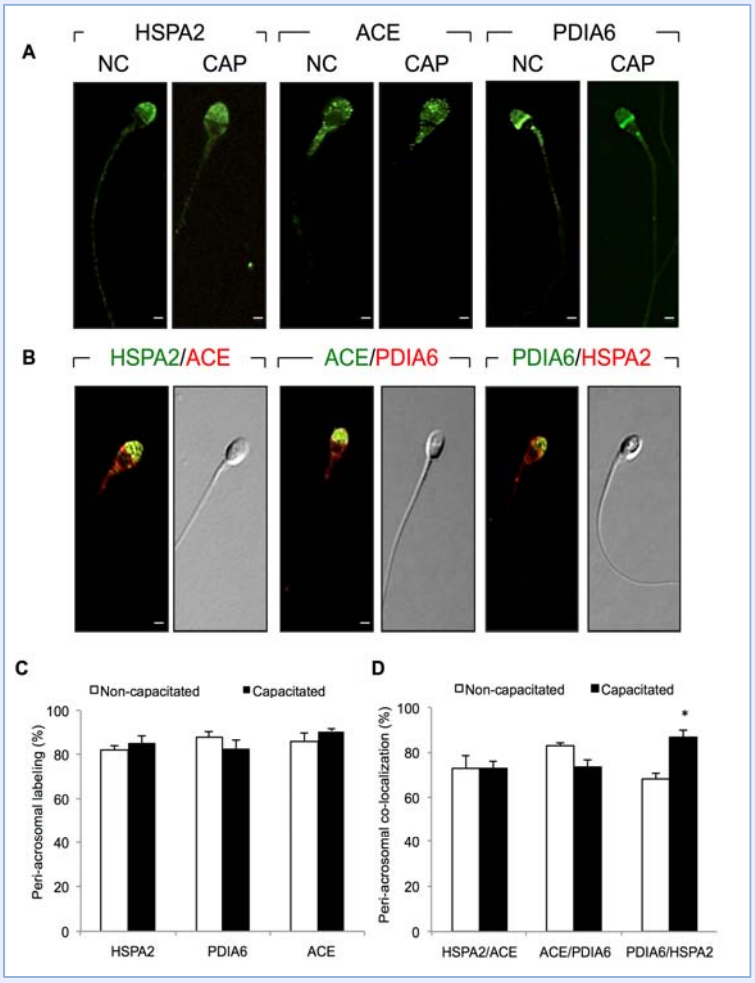

Figure 4: Immunolocalization of HSPA2, ACE and PDIA6 in non-capacitated and capacitated human spermatozoa. A) Immunolocalization of each protein of interest was performed with anti-HSPA2, anti-ACE and anti-PDIA6 antibodies on spermatozoa that had been held in either a non-capacitated state in a modified BWW medium lacking $NaHCO_3^-$, or in BWW supplemented with 25 mM $NaHCO_3^-$, 3mM pentoxifylline and 5mM dibutyryl cyclic AMP. B) The colocalization of these proteins was established in non-capacitated and capacitated spermatozoa by dual labelling with antibodies to HSPA2/ACE, ACE/PDIA6 and PDIA6/HSPA2 with representative images for capacitated spermatozoa displayed with their corresponding phase images. C) Labelling patterns were quantified by recording the percentage of spermatozoa that stained positively for HSPA2, ACE and PDIA6 across the peri-acrosomal head with 100 cells examined per slide. (D) Colocalization of HSPA2/ACE, ACE/PDIA6 and PDAI6/HSPA2 was quantified in capacitated spermatozoa with 100 cells per slide scored for peri-acrosomal co-localization of each set of proteins. Images were taken on a confocal microscope using a × 60 objective. * $P < 0.05$ between non-capacitated and capacitated cells. Scale bars = 3 μm.

Furthermore, dual- labelling experiments revealed overlapping expression patterns of each of these proteins within this peri-acrosomal domain (Figure 4B). Such labelling was present in >65% of the spermatozoa and was not influenced by their capacitation status (Figure 4D). Interestingly, the peri-acrosomal labelling of PDIA6 was also accompanied by an additional foci of intense staining within the equatorial region of non-capacitated and capacitated spermatozoa. Labelling of the sperm midpiece was consistently observed for HSPA2, ACE and PDIA6 in non-capacitated and capacitated spermatozoa with faint HSPA2 and PDIA6 labelling also detected in the tail.

To further visualize the interaction between these proteins *in-situ* a Proximity Ligation Assay (PLA) was used (Figure 5). PLA signal, detected as punctate red fluorescence, was observed across the head of non-capacitated and capacitated spermatozoa (counterstained with DAPI) that were interrogated with coupled antibodies; anti-HSPA2 and anti-ACE; anti-ACE and anti-PDIA6; anti-HSPA2 and anti-PDIA6 with their corresponding PLA probes (Figure 5A). Interaction between HSPA2 and ACE appeared primarily restricted to the peri-acrosomal region of the head, with similar findings for PDIA6 and HSPA2. While peri-acrosomal PLA labelling was also detected for ACE and PDIA6, additional staining was observed in the post-acrosomal and equatorial region, possibly reflecting the localization of PDIA6 observed in Figure 4. Importantly, the specificity of all PLA reactions was confirmed by incubating antibodies against each putative HSPA2-binding protein with an irrelevant antibody (anti-tubulin; Figure 5B).

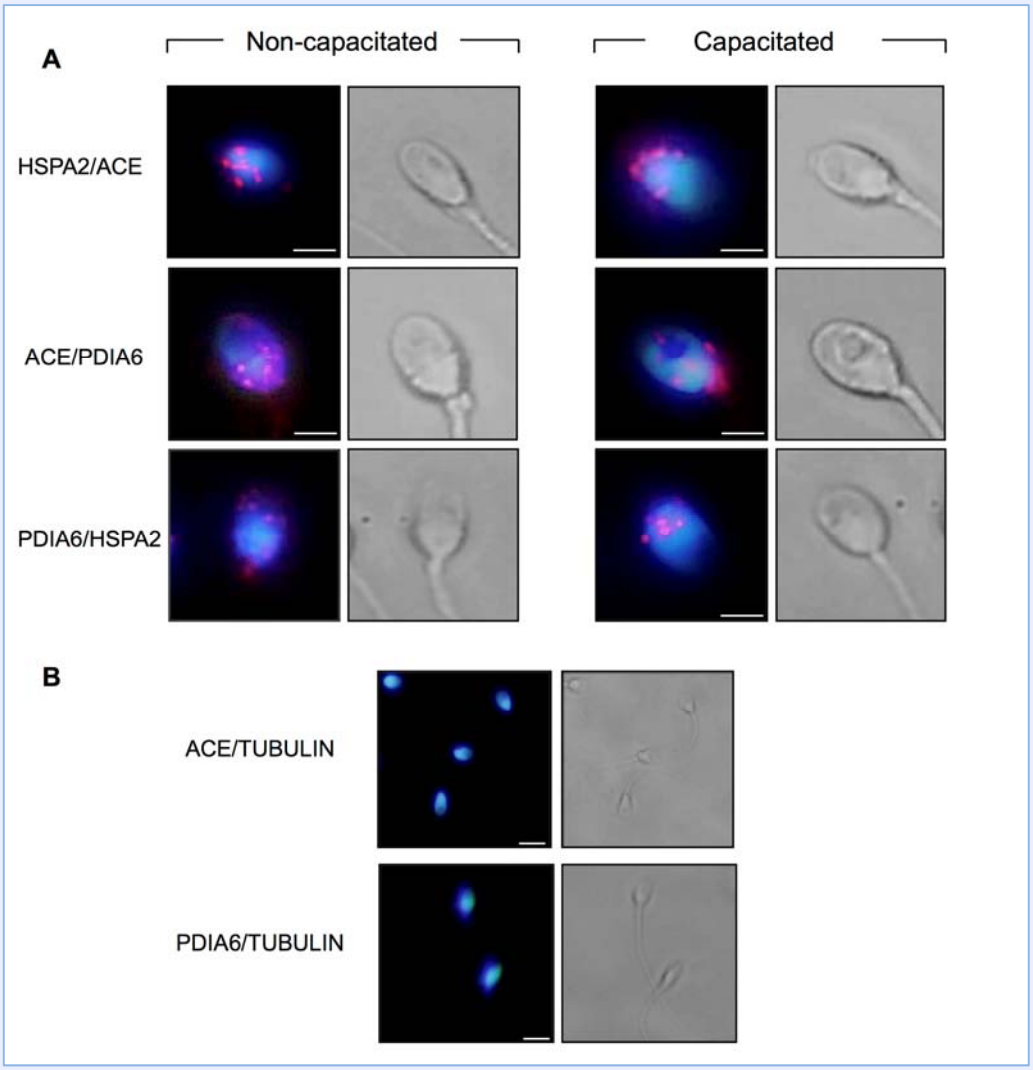

Figure 5: Proximity ligation of HSPA2, ACE and PDIA6 in human spermatozoa. (A) A proximity ligation assay was used to further confirm the interactions between HSPA2, ACE and PDIA6 in human spermatozoa. This assay results in the production of punctate red fluorescent signals when proteins of interest reside within a maximum of 40 nm from each other. For clarity, spermatozoa were counterstained with DAPI. As shown in (A), positive PLA signals, appearing as a number of discrete red fluorescent spots, were detected in the head of both non-capacitated and capacitated sperm populations using dual labeling with antibodies to HSPA2, ACE, PDIA6 and their corresponding oligonucleotide-conjugated secondary antibodies (PLA probes). The fluorescence signal was visualized using fluorescence microscopy using a ×100 objective, scale bars = 3μm. (B) To verify the specificity of this signal, anti-PDIA6 or anti-ACE antibodies were used in combination with anti-tubulin (an unrelated protein) and corresponding PLA probes. This resulted in no PLA signals observed in either non-capacitated or capacitated spermatozoa. Images were taken using a ×40 objective lens, scale bars = 3μm.

Examination of HSPA2-binding proteins in membrane rafts

Given the conserved localization of these proteins in the peri-acrosomal region of the sperm head, we were interested in determining whether their positioning may be influenced by their inclusion in membrane microdomains or 'rafts' within the human sperm head. Indeed, our previous work has identified both ACE and HSPA2 within the detergent resistant membrane fraction of human spermatozoa (Nixon et al., 2011) and PDIA6 present in analogous fractions in mouse spermatozoa (Nixon et al., 2009). Consistent with our previous findings we were able to demonstrate a capacitation-associated re-localization of membrane rafts (Nixon et al., 2011; Tanphaichitr et al., 2015), whereby the uniform distribution of CTB across the sperm flagellum and anterior head in non-capacitated cells, was replaced with a restricted pattern of raft labelling primarily within the peri-acrosomal head and midpiece of capacitated spermatozoa (Figure 6). Co-localization of ACE, PDIA6 and HSPA2 with the membrane raft marker of CTB confirmed the presence of each protein within the membrane raft enriched region of the peri-acrosomal sperm head of capacitated cells (Figure 6).

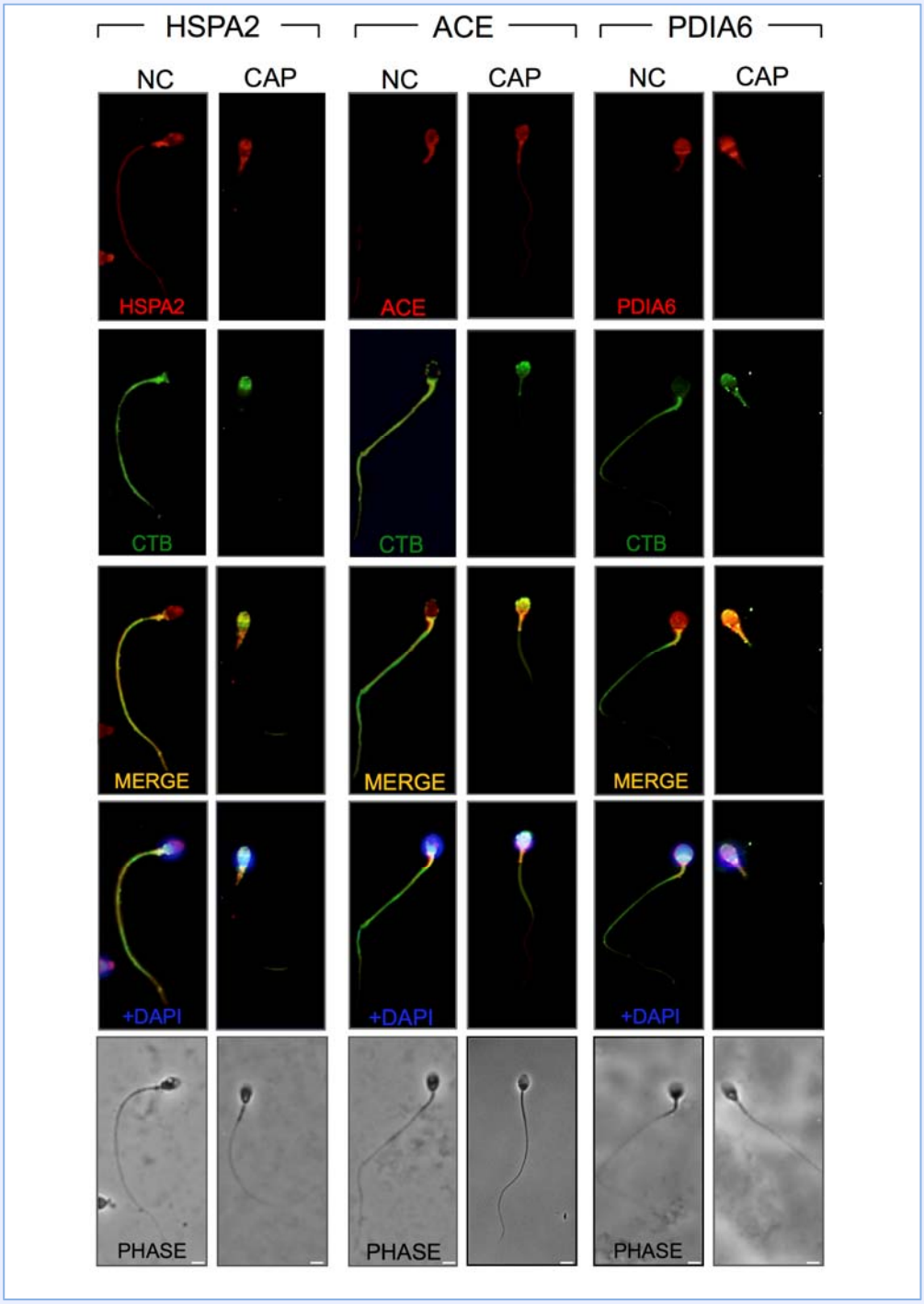

Figure 6: Co-localization of HSPA2, ACE and PDIA6 with the membrane raft marker Cholera toxin B (CTB) in human spermatozoa. Non-capacitated and capacitated spermatozoa were labelled with Alexa fluor 488 conjugated CTB prior to fixation and the subjected to immunocytochemistry with anti-HSPA2, anti-ACE and anti-PDIA6 antibodies and counterstained with DAPI (+DAPI). Results confirmed the redistribution of CTB-labelled membrane rafts during capacitation resulting in a coalescence of these membrane microdomains in the sperm head. Colocalization revealed the presence of HSPA2, ACE and PDIA6 within these CTB labelled regions of the sperm head after capacitation (MERGE). Fluorescence and phase images were recorded using a ×40 objective, scale bars = 3 µm.

Examination of the surface dynamics of ACE and PDIA6 in human spermatozoa

In terms of the extracellular location of our proteins of interest, the use of a live cell labelling assay (Figure 7) produced results consistent with our previous studies suggesting that HSPA2 is not detectable on the sperm surface under our non-capacitating or capacitating conditions. Similarly, ACE labelling of live cells revealed a small subset of non-capacitated cells with surface labelling (15%), and again this profile was not significantly influenced by the capacitation status of these cells. In marked contrast, PDIA6 labelling of spermatozoa revealed a marked increase in detectable surface fluorescence after capacitation with 7% of non-capacitated cells and 72% of capacitated cells displaying PDIA6 surface expression ($P < 0.001$; Figure 7A).

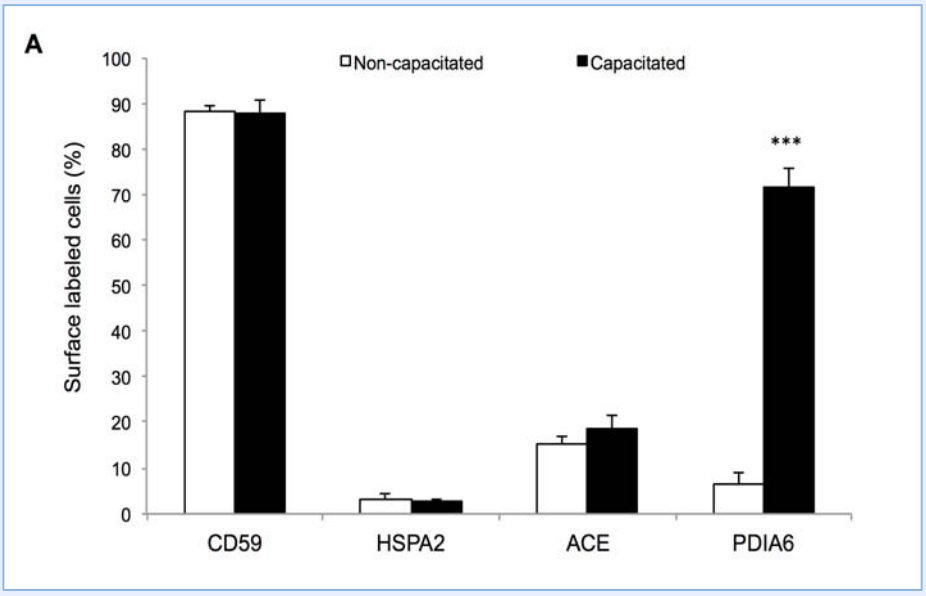

Figure 7A: Sperm surface analysis of HSPA2-client proteins. The presence of HSPA2, ACE and PDIA6 on the surface of live spermatozoa was assessed using appropriate primary antibodies in non-capacitated and capacitated spermatozoa, followed by Alexa Fluor-conjugated secondary antibodies and propidium iodide (PI) as a counterstain to assess cell viability. Positive control incubations were labeled with anti-CD59. The percentage of live sperm expressing surface fluorescence in each population was evaluated using a fluorescence microscope, scoring 200 cells across three biological replicates. Statistical analyses were performed using a Students t test, *** P< 0.001.

Interestingly, these data are analogous with our previous studies evaluating SPAM1 / ARSA complex dynamics, with the increased detectable surface expression of PDIA6 reminiscent of the increase in detectable ARSA surface expression shown previously (Redgrove et al., 2013). In a more recent study, we have demonstrated that this surface expression of ARSA could be perturbed by the induction of oxidative stress with H_2O_2 prior to capacitation (Bromfield et al., 2015b). Given this, we were interested in determining whether this phenomenon extended to other HSPA2-binding proteins.

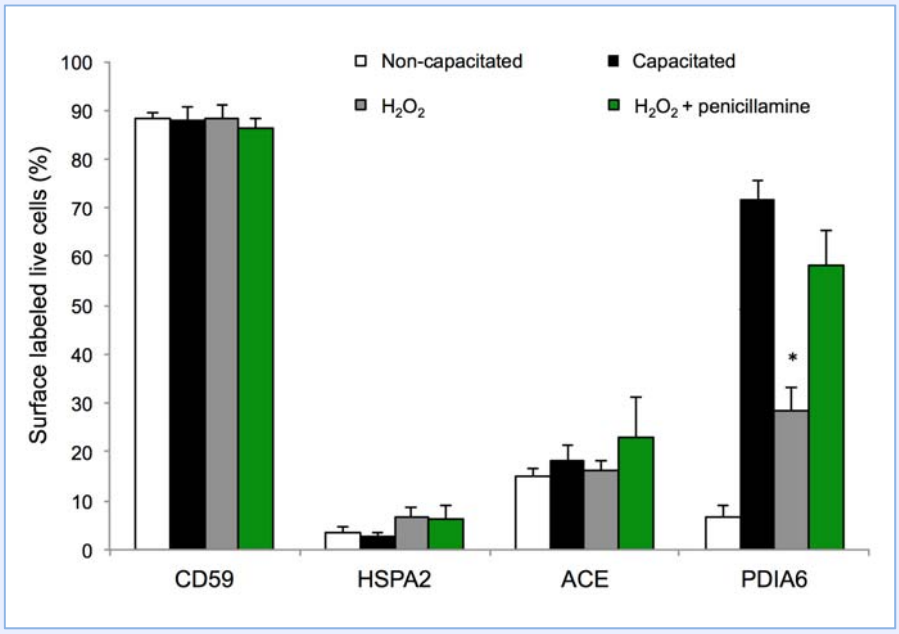

Figure 7B: Surface analysis of HSPA2-client proteins under conditions of oxidative stress.hb
Oxidative stress was induced in populations of human spermatozoa through treatment with 50μM hydrogen peroxide (H_2O_2) for 1 h. Treated spermatozoa were then washed once in non-capacitating BWW and then capacitated under standard capacitating conditions or in the presence of capacitating media supplemented with 1mM D-penicillamine. A population of non-capacitated cells was also prepared under standard non-capacitating conditions. The presence of HSPA2, ACE and PDIA6 on the surface of live spermatozoa was assessed by labelling with appropriate primary antibodies, followed by Alexa fluor-conjugated secondary antibodies and propidium iodide (PI) as a counterstain to assess cell viability. As a positive control, cells from each treatment were also labeled with anti-CD59. The percentage of live sperm expressing surface fluorescence in each population was evaluated using a fluorescence microscope, scoring 200 cells across three biological replicates. Statistical analyses were performed using a Students t test, * $P < 0.05$, compared to capacitated cell population labelled with PDIA6. This assay showed that PDIA6 surface expression was significantly reduced by treatment with H_2O_2 but this effect could be attenuated by the inclusion of penicillamine during sperm capacitation.

Pre-treatment of spermatozoa with 50μM hydrogen peroxide (H_2O_2) prior to capacitation resulted in a significant reduction of detectable PDIA6 surface labelling (Figure 7B; $P < 0.05$). Comparable to our previous data, inclusion of the nucleophile penicillamine in capacitating media was able to significantly reduce the effects of H_2O_2 on PDIA6 surface labelling resulting in statistically similar levels of PDIA6 to the capacitated control ($P > 0.05$). This is likely to be attributable to the lipid peroxidation product scavenging ability of penicillamine (Aitken et al., 2012). Notably, these treatments did not augment the expression of either ACE or HSPA2.

The effect of ACE inhibition on sperm capacitation

In view of our evidence for the stable interaction between HSPA2, ACE and PDIA6 in both developing and mature germ cells we next aimed to investigate a role for this complex in sperm function. In the absence of specific pharmacological inhibitors that target PDIA6, we instead elected to focus on the impact of ACE inhibition using the highly selective ACE inhibitor, captopril.

For these studies, sperm were capacitated in media supplemented with concentrations of captopril (25 μM – 200 μM) that have previously been shown to elicit potent ACE inhibition *in vitro* (Foresta et al., 1991). Regrettably, a lack of homologous human ZP prevented us from directly evaluating the effect of captopril on sperm interaction with, and/or penetration of, the zona pellucida. However, as shown in Figure 8A-C, even the highest concentrations of captopril had no overt effects on the progression of human spermatozoa through the *in vitro* capacitation process. Specifically, no significant effect of captopril was seen on sperm motility (Figure 8A) and similarly, staining of spermatozoa with Merocyanine 540 and phosphotyrosine revealed that captopril treatment did not compromise capacitation-associated increases in either membrane fluidization or the signalling pathways underpinning tyrosine

phosphorylation, respectively (Figure 8B-C). Indeed, we documented a significant, 6-fold increase in membrane fluidity and a ~60% increase in anti-phosphotyrosine labelling of the sperm flagella, a result characteristic of sperm capacitation, in either the presence or absence of captopril (Figure 8B-C).

Notwithstanding these results, spermatozoa capacitated in the presence of captopril proved refractory to calcium ionophore challenge. Indeed captopril treatment elicited a potent, dose-dependent inhibition of agonist (A23187) induced acrosome reaction rates (Figure 8D). At the highest dose of 200 μM captopril only 13% of capacitated spermatozoa proved capable of undergoing acrosomal exocytosis compared to 43% in the capacitated control ($P < 0.01$). Such data suggest that at least one function of the HSPA2, ACE, PDIA6 characterized in this study is modulation of the acrosomal responsiveness of human spermatozoa.

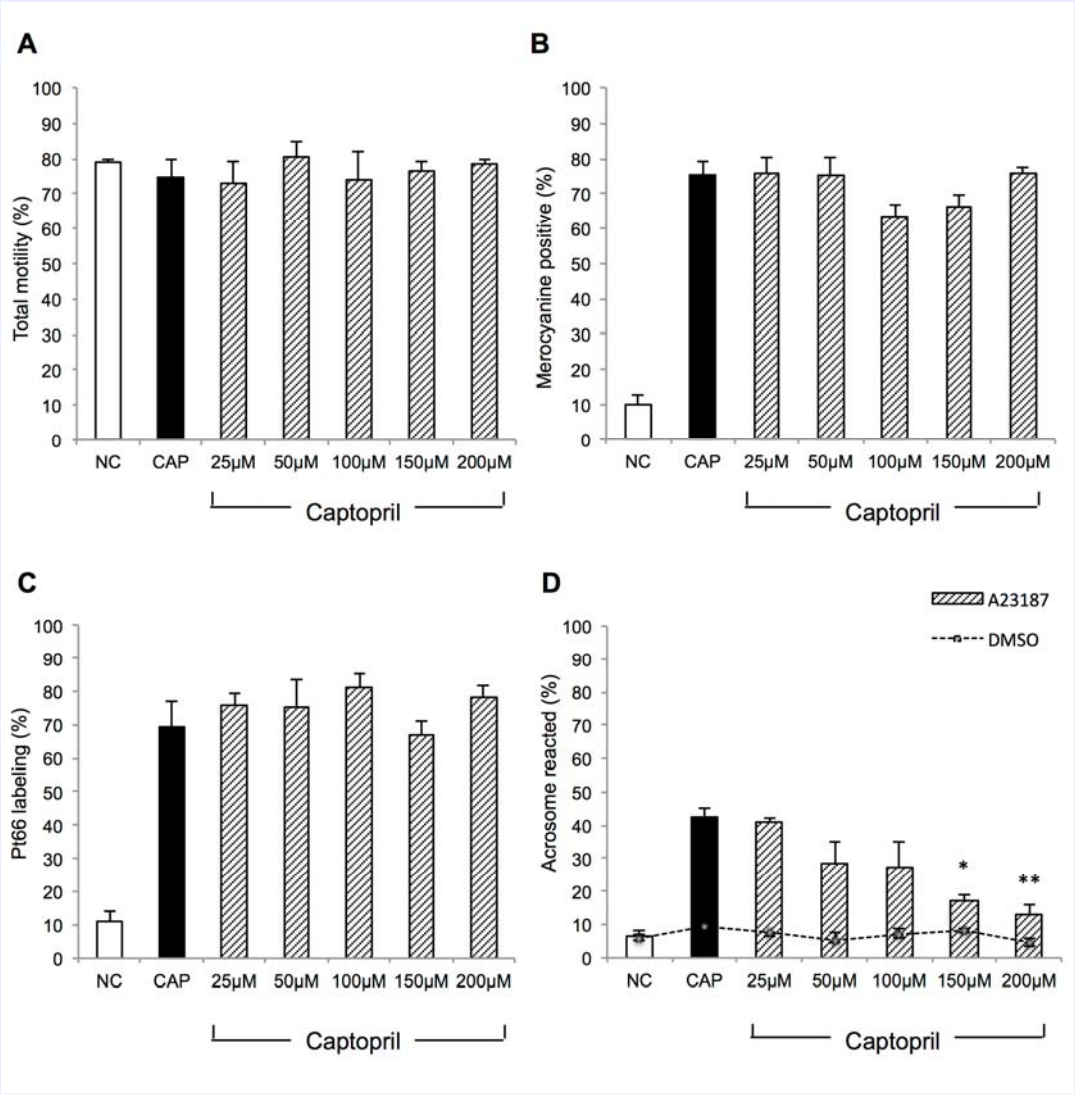

Figure 8: The effect of ACE inhibition on sperm motility, capacitation and an agonist induced acrosome reaction. Human spermatozoa were incubated under non-capacitating (NC) or capacitating conditions (CAP) or in capacitating media supplemented with 25-200 μM captopril, an inhibitor of ACE. The percent total motility of these cells was then evaluated using phase-contrast microscopy (A). (B) An aliquot of live spermatozoa from each treatment was stained with Merocyanine 540 and Sytox green to assess membrane destabilization/fluidity and cellular viability, respectively. Cells were then assessed for membrane fluidity by recording the proportion of 100 viable cells that displayed red Merocyanine 540 fluorescence across the sperm head. (C) An aliquot of cells from each treatment were fixed in 4% paraformaldehyde and incubated with a FITC-conjugated antibody to phosphotyrosine (α-PT66). Cells were scored as phosphotyrosine positive if they displayed complete labelling of the flagella with a total of 100 cells from each treatment evaluated. (D) Following capacitation, sperm from CAP and captopril-treated populations were treated with 1.25μM calcium ionophore (A23187) to induce acrosomal exocytosis or a DMSO control. Acrosome reacted cells were recorded through the use of TRITC labelled Peanut Lectin Agglutinin (PNA). An absence of PNA labelling over the acrosomal region indicated an acrosome reacted cell. Statistical analyses were performed using a Students t test, * $P < 0.05$, ** $P < 0.01$, compared to CAP.

DISCUSSION

Complete fertilization failure occurs in ~5-15% of conventional IVF treatments (Li et al., 2014) and while this lesion is undoubtedly attributed to a number of factors, defects in sperm-egg recognition are known to be a major underlying aetiology (Liu and Baker, 2000; Li et al., 2014). While a highly predictive test for fertilization success remains to be developed, the potential use of HSPA2 as a positive biomarker of fertilization success has been widely discussed (Cayli et al., 2003; Redgrove et al., 2012; as reviewed by Nixon et al., 2015). More recently, the germinal isoform of ACE has also been reported to have predictive value for human IVF success (Li et al., 2014). Specifically, spermatozoa of patients that have failed fertilization or have a low fertilization rate are commonly deficient in tACE expression and this may be causally related to a TT genotype in the rs4316 single-nucleotide polymorphism of tACE (Li et al., 2014). Excitingly, in the current study we provide the first evidence that HSPA2 and ACE form a stable interaction in human spermatozoa, which appears to be initiated in the post-meiotic germ cells of the testis.

ACE is perhaps best known for its role in the renin-angiotensin system where it is responsible for the cleavage of angiotensin I to the vasoconstrictor angiotensin II (Skeggs, 1993). However, it is becoming increasingly clear that the protein has a diverse range of additional biological functions, including renal development, modulation of immune response and the aetiology of many disease states such as nephropathy, heart failure and hypertension (as reviewed by Bernstein et al., 2013). In addition, somatic ACE consists of two homologous catalytic domains (N- and C-terminal domains) that allow it to process a diverse range of substrates through its carboxypeptidase activity (Skidgel and Erdos, 1987; Masuyer et al., 2014). By comparison, tACE has a unique 66 amino acid N-terminal sequence and hence possesses only a single catalytic domain, despite the C-terminal portion being identical to somatic ACE (Howard et al., 1990). In an elegant transgenic mouse model featuring the

substitution of tACE with sACE it was clearly shown that the two isoforms are functionally non-equivalent. Indeed, the severe subfertility associated with tACE ablation could not be rescued by sACE expression (Kessler et al., 2000).

In the present study, we identified a novel association between ACE, HSPA2 and the protein disulfide isomerase, PDIA6 (previously ERP5), in a single protein complex in human spermatozoa. The combined molecular weight of this complex suggests that it specifically incorporates the tACE isoform. The presence of a higher molecular weight band that likely corresponds to the sACE monomer may be due to the abundance of sACE in seminal plasma. As suggested by Nikolaeva and colleagues, somatic ACE can passively absorb to the surface of spermatozoa and thus it is difficult to assess the presence of tACE in a given cell lysate without contamination of sACE (Nikolaeva et al., 2006).

Our localization studies of ACE, HSPA2 and PDIA6 in human testis tissue suggest that an interaction between these proteins is initiated in testicular germ cells. All three proteins co-localized within the later-stage germ cells of the testis, consistent with previous studies suggesting that HSPA2, ACE and PDIA6 each fulfil important functions in post-meiotic germ cells (Govin et al., 2006; Fujihara et al., 2013; Van Lith et al., 2007). In the mouse, quality control (QC) complexes, such as those comprising calreticulin (CALR3) and the protein disulfide isomerase homolog (PDILT) in the testis (Tokuhiro et al., 2012), or Protein disulfide isomerase 3 (PDIA3) and calnexin (CANX) in somatic cells (Antoniou et al., 2002), are employed to ensure appropriate protein function. As in the case of ADAM3 processing, such testicular QC complexes regulate dilsulfide bonding and direct glycoprotein folding in the endoplasmic reticulum and thus appear to be crucial in equipping spermatozoa with their fertilizing ability (Tokuhiro et al., 2012). The co-localization of PDIA6 and HSPA2 in the testis leads us to speculate that these two proteins may be components of similar machinery in the human and that their cooperative function may be important for the

processing of polypeptides implicated in fertilization. Interestingly, ACE is heavily reliant upon such processing to maintain its dimerized form via a 'disulfide zipper' consisting of disulfide bonds between six highly conserved cysteines (Sturrock et al., 1996). ACE has been characterized as a redox sensor as it is highly resistant to redox reduction (Moskovitz and Johnson, 2004) and exists predominantly in its oxidized form. Maintenance of this oxidized state is likely to require the concerted action of protein disulfide isomerases such as PDIA6 (Ellgard and Ruddock, 2005).

In mature spermatozoa, HSPA2, ACE and PDIA6 co-localization was observed predominantly, although not exclusively, in the peri-acrosomal region of the head. Due to the presence of a J-domain in protein disulfide isomerases, they have previously been predicted to cooperate with alternative chaperones of the HSP70 family in the testis (Cunnea et al., 2003; Hosoda et al., 2003). However, to the best of our knowledge this is the first report of an interaction between PDIA6 and HSPA2 in mature spermatozoa.

Consistent with their involvement in a single protein complex in human spermatozoa, each of the three target proteins were shown to co-localize with a well-defined marker of membrane rafts within the anterior region of the sperm head. These microdomains are commonly laden with molecular chaperones (Nixon et al., 2009; 2011; Tanphaichitr et al., 2015) and have an important role in the translocation and/or clustering of receptor proteins in the sperm head in preparation for their interaction with the ZP. It is therefore tempting to speculate that the raft domains may provide a platform to assist with the recruitment of HSPA2-interacting proteins and thus assembly of important multimeric complexes in the anterior region of the human sperm head (Redgrove et al., 2011; 2012; reviewed by Bromfield and Nixon, 2013). Certainly, such positioning would be crucial for ACE to fulfill its putative role in the cleavage of glycophosphatidylinositol (GPI)-anchored proteins, such as TEX101 (Fujihara et al., 2013). In addition, the clustering of membrane rafts and their constituent

receptor proteins is known to be an efficient means of signal regulation (Simons and Toomre, 2000). In this context, it is well known that ACE plays a key role in the regulation of several signalling pathways (Santhamma and Sen, 2000; Fleming, 2006; Kohlstedt et al., 2006).

In mouse spermatozoa, the primary role of tACE appears to rest with its ability to regulate the processing of ADAM3 prior to sperm-egg interaction (Yamaguchi et al., 2006; Fujihara et al., 2013; 2014). However, given that human spermatozoa do not express an ADAM3 orthologue (Frayne and Hall, 1998; Okabe, 2015), determining the function of tACE in these cells has proven to be an ongoing challenge with a number of opposing models being actively debated (as reviewed by Bernstein et al., 2013).

In recent studies, the GPIase activity of tACE has been implicated in the shedding of GPI-anchored proteins such as SPAM1 and TESP5 on the sperm surface prior to contact with the ZP (Kondoh et al., 2005). Interestingly, the loss of SPAM1 from the human sperm surface supports our previous observations that this hyaluronidase shows a decrease in surface expression after capacitation (Redgrove et al., 2012). The loss of this intrinsic GPI-ase activity in ACE null mice is proposed to account for the loss of ZP binding ability observed in these mice and hence their infertility (Kondoh et al., 2005). However, the interpretation of these results has drawn criticism owing to the fact that ACE is unable to access GPI-anchored proteins without prior disruption of membrane rafts using filipin. This has led to the proposal that the infertility of male ACE null mice can be attributed to the loss of the dipeptidase activity of ACE (Leisle et al., 2005; Fuchs et al., 2005). However, it should also be considered that the use of exogenous ACE to verify its GPIase activity may result in issues of sterical hindrance that can only be overcome by the use of filipin, an issue that is not apparent for intrinsic ACE. Alternatively, the depletion of cholesterol during physiological capacitation, or indeed that induced by filipin, may be required for the induction or ACE activity. Interestingly, despite its purported roles in the mouse, inhibition of ACE in human

spermatozoa did not appear to result in impaired sperm-ZP interaction but did result in reduced penetration of the oolemma of ZP-free hamster oocytes (Kohn et al., 1998).

Using monoclonal antibodies to ACE, the surface expression of this protein has been previously reported in ejaculated human spermatozoa (Nikolaeva et al., 2006). Despite this, our examination of protein surface expression revealed that only a modest subset of the sperm population present ACE on their surface. This result does not appear to support the hypothesis that ACE is solely responsible for the loss of GPI-anchored proteins. However, ultrastructural studies have indeed localized ACE to the acrosomal, equatorial and post-acrosomal regions of the sperm head plasma membrane (PM), although this study did not quantify the proportion of the sperm population possessing surface tACE (Kohn et al., 1998). Additionally, a pool of immunoreactive ACE was found on the outer acrosomal membrane of PM-compromised cells (Kohn et al., 1998). In somatic cells, the interaction of ACE with HSPA5, a chaperone of the HSP70 family, has been shown to regulate its expression at the plasma membrane (Santhamma and Sen, 2000). While it may be possible that HSPA2 plays a similar role in regulating ACE at the sperm surface, no obvious change in the localization of ACE was observed over the course of the capacitation process during which HSPA2 is likely to be activated by phosphorylation (Redgrove et al., 2013).

Conversely, detectable PDIA6 surface fluorescence dramatically increased in the capacitated sperm population. In this light, the dynamic reshuffling of existing surface proteins after sterol loss, combined with the orchestrated removal of other surface proteins during capacitation may result in the unmasking and/or presentation of PDIA6 epitopes that may be more readily detected by our antibodies. While this may suggest that the positioning of PDIA6 on the cell surface could be modified during capacitation, ultrastructural studies are required to confirm this finding and rule out the possibility that the early events of the

acrosome reaction may lead to the exposure of some sperm proteins. Moreover, our observations of changes in PDIA6 surface fluorescence after the addition of the pro-oxidant H_2O_2 and the anti-oxidant penicillamine may suggest the importance of the redox status of a subset of cell surface proteins to ensure their correct alignment or partitioning into functional complexes.

While the function of PDIA6 in human spermatozoa requires specific investigation, PDI proteins have been shown to regulate cell-cell adhesion as well as maintain the reductive status of the plasma membrane (Turano et al., 2002). Indeed, proteins with disulfide isomerase activity have been widely implicated in regulation of the extensive conformational changes experienced by many elements of the fusion machinery before realising their potential to facilitate cell-cell fusion (Sanders, 2000; Hogg, 2002; Matthias et al., 2002; Dun et al., 2012). Of particular note, two of the candidate proteins implicated in gamete fusion, IZUMO1 on the sperm surface and CD9 on the egg plasma membrane, are known to possess disulfide residues in their extracellular domains (Ellerman et al., 2006). Moreover, evidence for the participation of PDI family members in gamete fusion was uncovered in the mouse model through the use of broad-spectrum PDI inhibitors which elicited a potent inhibition of gamete fusion (Ellerman et al., 2006). These studies also confirmed the presence of PDIA3 on the mouse sperm surface (Ellerman et al., 2006). While we are unable to perform similar assays to evaluate human sperm-egg fusion, the surface expression of PDIA6 in human spermatozoa makes it a promising candidate for the regulation of cell adhesion and fusion processes in our own species.

The final series of experiments of this study focused on the use of the ACE inhibitor captopril during human sperm capacitation. Captopril binds to ACE through interaction with its thiol group leading to the formation of a zinc (II)-thiolate complex (Krassnigg et al., 1986).

The resulting adduct compromises the ability of ACE to participate in substrate binding. In this study we demonstrated that such ACE inhibition did not have a significant effect on sperm motility, membrane fluidity or protein tyrosine phosphorylation, suggesting that this protein is unlikely to play a key role in the regulation of capacitation. However, at the doses examined, captopril did suppress the ability of sperm to undergo agonist induced acrosomal exocytosis. This finding is in agreement with reports by Foresta and colleagues who have previously suggested that ACE plays a role in the completion of the acrosome reaction (Foresta et al., 1991). Additionally, these authors observed a decrease in oocyte penetration rates when human spermatozoa were treated with captopril. Moreover, the localization of ACE to the outer acrosomal membrane of plasma membrane-compromised spermatozoa (Kohn et al., 1998) and the peri-acrosomal labelling of ACE observed in our study are both indicative of that the protein is suitably positioned for a role in modulating acrosome stability. This also conforms with studies in other species such as the pig, where ACE has been identified in the anterior head plasma membrane (Tanphaichitr et al., 2015).

Potential mechanism(s) by which ACE may regulate the acrosome reaction are numerous. Firstly, ACE has been reported to exhibit signalling behaviour through its ability to bind both PKC and calmodulin (Santhamma and Sen, 2000; Chattopadhyay et al., 2005; Fleming, 2006). In this way, interfering with ACE signalling may affect calcium second messengers that are required for activation of key acrosomal proteins (Breitbart et al., 1992). Furthermore, the key product of ACE activity, angiotensin II, is known to activate MAP kinases, tyrosine kinases and metalloproteinases (Sachse and Wolf, 2007) as well as regulate intracellular calcium (Diaz-Torga et al., 1998). The inhibition of angiotensin II would therefore be expected to have a negative effect on the acrosome reaction. However, an important caveat to these findings is that subfertility in men taking ACE inhibitors for hypertension has not been reported. While this may be explained by differences in the doses

examined/administered, it does imply that captopril may work via an alternative mechanism to suppress sperm function. It is therefore clear that the role of ACE in the human sperm acrosome reaction needs to be evaluated in a more physiological setting before definitive conclusions can be drawn.

In conclusion, this study has demonstrated that ACE and PDIA6 are likely substrates of the molecular chaperone HSPA2 and form a stable interaction in the germ cells of the testis that persists into fully mature human spermatozoa. We have provided evidence for the partitioning of these proteins into the membrane raft components of the peri-acrosomal sperm head and preliminary evidence for the involvement of ACE in modulating agonist induced acrosomal exocytosis. As our research group, and others, has shown that HSPA2 is compromised in the spermatozoa of men with oocyte recognition defects, the characterization of these HSPA2-interacting proteins provides important insight into the complexity of the cellular pathways that may be affected in the spermatozoa of these infertile individuals.

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Author contributions

E.B contributed to study design, conducted the experiments and generated the manuscript. R.J.A. and E.A.M. contributed study design and data interpretation. B.N. contributed to study conception and design, data interpretation and manuscript preparation.

Conflict of Interest

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

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CHAPTER 5: FINAL DISCUSSION

The role of the molecular chaperone heat shock protein A2 (HSPA2) in regulating human sperm-egg recognition

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Chapter 5: Overview

The final chapter of this thesis provides an overview of the specific function of HSPA2 in the regulation of human sperm-egg recognition. Herein, the advances made by our studies on the role of HSPA2 in sperm function are summarized and several mechanisms are proposed to explain how HSPA2 expression may be compromised in the spermatozoa of infertile individuals. Specifically, these mechanisms include: genetic mutations in the encoding *Hspa2* gene, epigenetic regulation of *Hspa2* gene expression, and/or perturbations in protein expression/stability arising from exposure of developing germ cells to oxidative stress. With regard to the latter it is possible the oxidative attack could act directly to damage the *Hspa2* gene or mRNA transcript. Alternatively, as supported by the studies in this thesis, the HSPA2 protein itself may be targeted for destruction following adduction by electrophilic aldehydes generated as a result of reactive oxygen species-induced lipid peroxidation. Finally, this manuscript highlights the predictive value of HSPA2 for the success of both IVF and ICSI.

The findings described in this thesis provide novel insight into the loss of oocyte recognition-ability in human spermatozoa. These advances have developed our understanding of the extent by which molecular chaperones participate in the formation and function of functionally competent spermatozoa and highlight the vulnerability of the male germ-line to oxidative stress.

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INVITED REVIEW

Sperm Biology

The role of the molecular chaperone heat shock protein A2 (HSPA2) in regulating human sperm-egg recognition

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One of the most common lesions present in the spermatozoa of human infertility patients is an idiopathic failure of sperm-egg recognition. Although this unique cellular interaction can now be readily by-passed by assisted reproductive strategies such as intracytoplasmic sperm injection (ICSI), recent large-scale epidemiological studies have encouraged the cautious use of this technology and highlighted the need for further research into the mechanisms responsible for defective sperm-egg recognition. Previous work in this field has established that the sperm domains responsible for oocyte interaction are formed during spermatogenesis prior to being dynamically modified during epididymal maturation and capacitation in female reproductive tract. While the factors responsible for the regulation of these sequential maturational events are undoubtedly complex, emerging research has identified the molecular chaperone, heat shock protein A2 (HSPA2), as a key regulator of these events in human spermatozoa. HSPA2 is a testis-enriched member of the 70 kDa heat shock protein family that promotes the folding, transport, and assembly of protein complexes and has been positively correlated with *in vitro* fertilization (IVF) success. Furthermore, reduced expression of HSPA2 from the human sperm proteome leads to an impaired capacity for cumulus matrix dispersal, sperm-egg recognition and fertilization following both IVF and ICSI. In this review, we consider the evidence supporting the role of HSPA2 in sperm function and explore the potential mechanisms by which it is depleted in the spermatozoa of infertile patients. Such information offers novel insights into the molecular mechanisms governing sperm function.
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Keywords: egg; fertilization; heat shock protein A2; molecular chaperone; sperm; sperm-egg interactions

INTRODUCTION

Male infertility afflicts at least 1 in 20 men of reproductive age.[1] Notwithstanding a small percentage of male patients who exhibit azoospermia,[2,3] the majority of infertile men produce sufficient numbers of spermatozoa to fertilize an ovum *in vivo*. However, the quality of these individuals' gametes is compromised to the point that fertilization and the initiation of normal embryonic development are not possible. One of the most frequent functional defects in these cells is an inability to recognize and adhere to the outer vestments of the egg, a structure known as the zona pellucida (ZP), and subsequently engage in the complex cascade of cellular processes that culminate in fertilization.[4] Accordingly, bioassays of sperm-ZP interaction can accurately predict male infertility *in vivo*.[5] Indeed, assessment of sperm-ZP binding with the hemizona assay provides the highest discriminatory power for fertilization success/failure of any sperm parameter assessed.[6,7]

Despite the biological importance of ZP binding, the fact that this barrier can now be readily breached through the advent of assisted reproductive technologies such as intracytoplasmic sperm injection (ICSI) has meant that the molecular basis of sperm-ZP recognition remains poorly characterized.[8] This is particularly alarming in view of large-scale epidemiological studies that have documented an increased risk of birth defects in children conceived via ICSI, but not necessarily by conventional *in vitro* fertilization.[9] Such findings raise the prospect that the human ZP may possess the ability to select superior quality spermatozoa, a notion supported by recent demonstrations that the ZP selectively binds sperm with normal morphology and nuclear chromatin DNA.[10] Furthermore, biological selection of sperm for ICSI on the basis of their ZP binding affinity has been shown to produce higher quality embryos and contribute to improved implantation and clinical pregnancy rate compared to sperm selected by conventional subjective approaches.[11–13] Thus, in spite of the major advance ICSI has provided for the alleviation of male-factor infertility, there is a pressing need for basic research into physiopathology of sperm-ZP interactions.

Research into this cell-specific and tightly regulated interaction has revealed that it is coordinated by specialized sperm domains overlying the anterior region of the sperm head. These domains are formed during the latter phases of spermatogenesis before being dynamically modified upon passage through both the male and female reproductive tracts.[14] Thus, freshly ejaculated spermatozoa cannot recognize the egg; only after these cells have undergone a complex process of functional maturation, known as capacitation, do they express any affinity for the

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ZP.[15,16] The ZP ligands that mediate sperm-egg recognition are currently being actively debated, with models centered on the importance of ZP2 and/or ZP3/4 under consideration.[17–20] Similarly, the identity of the ZP receptor(s) on the surface of mammalian spermatozoa remains elusive. While a variety of candidates have been described, gene deletion studies have failed to confirm the exclusive significance of any of these molecules in mediating sperm-egg recognition.[21] An alternative concept founded on the basis of studies by Asquith *et al*.,[16] suggests that the biological importance of this event is so great that no single molecular entity has sole responsibility for mediating sperm-ZP binding. Rather, sperm-egg recognition is proposed to be a highly redundant process mediated by several ZP receptors that are brought to the cell surface and/or assembled into functional complexes during capacitation under the influence of molecular chaperones.[22–24]

Although this model was originally developed on the basis of studies conducted in the mouse, more recent work supports its relevance to ZP recognition in the human.[25,26] Among the chaperones that have been implicated in this process in human spermatozoa, a testis-enriched member of the heat shock protein (HSP) 70 family, HSPA2 (*Hspa2*), has emerged as a key candidate. In the current review, we consider the established and rapidly emerging roles of HSPA2 in promoting the morphological differentiation of the male gamete during spermatogenesis and the subsequent functional transformation of these cells during capacitation. Such data serve to highlight the potential of HSPA2 as a clinically useful marker of sperm quality and emphasize the need for further analysis of this chaperone as a means of providing important insights into some of the most challenging questions concerning the molecular mechanisms regulating sperm function.

BACKGROUND TO THE HSP70 FAMILY OF MOLECULAR CHAPERONES

Molecular chaperones constitute a large family of structurally diverse proteins that are ubiquitously expressed in all organisms.[27] More than 20 chaperone families, differing primarily with regard to their molecular weight and structural characteristics, have been described. Due to their ability to confer cellular resistance to environmental stressors, the majority of these chaperone families are referred to as cell stress response or, more commonly, HSPs.[28] In mammalian species, the HSPs are divided into the HSP100 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), HSP40 and HSP27 (HSPB) families.[29] Several members of each gene family are represented within the human genome,[30] a redundancy that may either relate to differences in intra-organelle compartmentalization and/or tissue/development-specific expression patterns.[31] The overlapping expression of many different molecular chaperone families highlights the importance of their specialized functions, which extend from archetypical protective roles through to regulation of normal cellular functions, including: metabolism, growth, differentiation and apoptosis.[32] Molecular chaperones can fulfill these diverse functions by virtue of their ability to selectively recognize and interact with exposed hydrophobic domains in their client proteins. Such interactions prevent inappropriate association or aggregation and direct the proteins into productive folding, transport or degradation pathways.[33]

The 70 kDa HSPs (HSP70) are among the most highly abundant and conserved members of the chaperone family, with at least 13 members represented in the human genome.[30] These folding catalysts possess a modular architecture comprising three major functional domains: a conserved N-terminal ATPase domain, a substrate-binding domain and a C-terminal domain that acts as a lid for the substrate binding domain.[34,35] The substrate binding and release cycle of HSP70s is commonly regulated by co-chaperones from the family of J-domain proteins (primarily HSP40 in eukaryotes) that target these chaperones to their respective substrates, and is further fine-tuned by nucleotide exchange factors.[36] The primary function of HSP70s centers on their ability to transiently bind to partially synthesized or denatured peptide sequences, thereby preventing their aggregation and allowing them to (re)fold into a functional state. However, by virtue of their ability to stabilize client proteins in a partially folded state, HSP70s also aid in the transmembrane transport of proteins, and in their assembly into functional complexes[35] (**Figure 1**). A novel, testis-enriched member of this HSP70 family, known as HSPA2, has emerged as a key regulator of several phases of sperm development and maturation.[26,37,38]

THE ROLE OF HEAT SHOCK PROTEIN A2 IN MOUSE SPERMATOZOA

Heat shock protein A2 (HSPA2) was originally identified in experiments designed to assess the effects of heat shock on protein synthesis in the germ cells of male mice.[39–41] Subsequent work revealed that *Hspa2* mRNA transcripts[42] and protein[43] displayed an expression profile that was both testis-enriched[44] and developmentally regulated.[45] Thus, gene expression was initiated in early meiosis[43,45] and immediately followed by protein synthesis in leptotene–zygotene spermatocytes.[46] Targeted mutation of the *Hspa2* gene[47] revealed that the chaperone is indispensable for the transition of spermatogenic cells through the late meiotic stages of spermatogenesis.[48] Specifically, it has been shown that *Hspa2* null males are infertile due to the combined effects of arrested spermatogenic cell development coinciding with the G_2–M-phase transition of meiosis I prophase and the apoptotic elimination of late stage pachytene spermatocytes.[48,49] Such a pronounced phenotype has been attributed to two primary roles for HSPA2 in these cells. Firstly, HSPA2 supports the formation of a heterodimeric complex between CDC2 and cyclin B1,[50] and secondly, HSPA2 appears to act as a component of the synaptonemal complex.[48] More recent work has

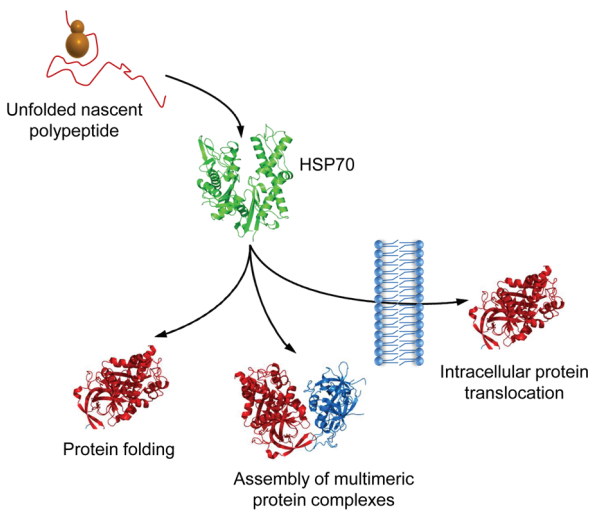

Figure 1: Functional roles of heat shock proteins (HSP). The evolutionarily conserved molecular chaperones of the HSP70 (HSPA) family fulfill an essential role in maintaining protein quality control in a variety of cell types. Such protective activities center on the ability of the chaperones to assist the correct (re)folding of nascent and denatured proteins, thereby preventing their unwanted aggregation and functional inactivation. However, HSP70s also play an important role in facilitating protein-protein interactions, the assembly of multimeric protein complexes, and in the transport of proteins across intracellular membranes.

shown that such functions may be augmented by the interaction of HSPA2 with an additional suite of testis enriched proteins, including: SHC SH2 domain-binding protein 1-like protein,[51] the nuclear autoantigenic sperm protein[52] and, the putative DExD-box helicase MOV10-like-1 that is essential for safeguarding the genetic information in the male germline.[53]

Interestingly, the stability of the HSPA2 protein during this critical phase of germ cell development is also influenced by its interaction with BAT3 (HLA-B associated transcript 3; also known as BCL2-associated athanogene 6, BAG6),[54] a chaperone-like protein that appears to be important for the folding and activity of apoptotic signaling molecules.[55] In this context, it has been shown that *Bat3* deficiency leads to the poly-ubiquitination and subsequent degradation of HSPA2 protein.[54] As anticipated, the loss of HSPA2 in *Bat3* deficient mice arrests meiosis at prophase I and induces apoptosis in late pachytene spermatocytes, thereby resulting in complete male infertility.[54] Such findings identify BAT3 as a critical regulator of HSPA2 in spermatogenesis and raise the prospect that it may represent a molecular target in idiopathic male infertility.

In addition to its fundamental roles in the completion of meiosis, the abundant expression of HSPA2 in postmeiotic germ cells has encouraged speculation that the protein fulfills additional function(s) during spermiogenesis. This notion is supported by evidence that, after the completion of meiosis, HSPA2 acquires a new role as a chaperone of spermatid-specific DNA packaging transition proteins.[38] These transition proteins serve as an intermediary, replacing histones before themselves being replaced by protamines during the nuclear condensation that accompanies spermiogenesis.[56] Owing to its ability to escort the transition proteins and mediate their assembly into DNA packaging structures, HSPA2 is thereby able to act as a major regulator of genome reorganization in differentiating spermatids.[38] Further studies have also implicated the chaperoning activity of HSPA2 in the correct folding, assembly or trafficking of the subunits comprising the CatSper ion channel that is required for sperm cell hyperactivation and male fertility.[57] Nevertheless, the HSPA2 protein has yet to be ascribed any specific functional role in mature mouse spermatozoa.

THE ROLE OF HEAT SHOCK PROTEIN A2 IN HUMAN SPERMATOZOA

Following its original identification in the mouse testes, immunoreactive HSPA2 protein homologs have since been reported in the testes of diverse phyla thus raising the prospect that it may play a highly conserved functional role during spermatogenesis.[37] In support of this concept, the human and mouse *Hspa2* homologs possess 91.7% identity in the nucleotide coding sequence and 98.2% in the corresponding amino acid sequence.[58] Examination of the human HSPA2 protein has revealed significant expression in normal testes, with immunoreactivity being detected in spermatocytes and spermatids.[59] Subsequent work confirmed that *Hspa2* is selectively expressed in a biphasic pattern during human spermatogenesis.[60] Thus, the first wave of *Hspa2* expression occurs in spermatocytes where it is predicted to support meiosis. In contrast, the second wave occurs in elongating spermatids during spermiogenesis.[60] Importantly, however, there is presently no direct evidence that human HSPA2 is involved in the dissociation of the synaptonemal complex or in the chaperoning of cyclin-CDK complexes as has been reported in the mouse.

Nevertheless, the importance of HSPA2 in the production of male germ cells has been highlighted by the demonstration that down-regulation of *Hspa2* gene expression is strongly correlated with significant reductions in sperm concentration. Indeed, in the case of both oligozoospermic ART patients[61] and those individuals suffering from complete azoospermia associated with spermatocyte arrest or Sertoli cell-only syndrome,[62] the relative levels of *Hspa2* gene expression are significantly lower than that of fertile controls. Similarly, aberrant HSPA2 protein expression has also been reported in immature human spermatozoa, which fail to complete normal spermiogenesis. This defect results in the production of spermatozoa with excessive cytoplasmic retention[60,63] and a reduced ability to engage in interactions with both the ZP and cumulus matrices.[64,65] Accordingly, HSPA2 has also been identified among a small number of proteins that are under-represented in defective spermatozoa with lesions in egg recognition.[26] However, a key difference of this latter comparative proteomic study was that it focused on a subset of infertile donors whose spermatozoa exhibited an isolated lesion in their ability bind to the ZP without any accompanying defects in sperm motility or morphology.

On the basis of these conflicting data, at least two models have emerged to account for the role of HSPA2 in promoting ZP binding and cumulus matrix penetration. The first of these has been pioneered by Huszar and colleagues who postulate that the chaperoning activity of HSPA2 (originally described as a variant of creatine kinase M) is restricted to sperm development within the testis where it is required to facilitate major cycles of protein transport that drive cytoplasmic extrusion and plasma membrane remodeling during spermiogenesis.[60,64–67] Such events are believed to not only underpin the formation of the ZP-binding domains but also those that are responsible for binding of the hyaluronic acid rich matrix of the cumulus mass. Consequently, the levels of HSPA2 remaining in mature human spermatozoa and the capacity of these cells to bind hyaluronic acid polymers, have both been reported to provide a robust discriminative index for fertilizing potential.[64,65,68]

An alternative model suggests that HSPA2 may instead play an important functional role in mature spermatozoa following their morphological differentiation within the testes. This model draws on evidence that HSPA2 is retained in mature spermatozoa and is ideally positioned in the peri-acrosomal region to participate in oocyte interactions.[26] However, this model is not without controversy given that the protein has been variously reported to be constitutively expressed on the human sperm plasma membrane,[69] to undergo a significant, albeit modest, increase in surface expression following the induction of capacitation in spermatozoa from fertile donors (i.e., 6.15% ±1.35 *vs* 13.1% ±2.69; $P = 0.017$),[70] or to remain permanently within an intracellular location.[25,26] Whether such controversy simply reflects the use of different methods of detection and/or antibodies[71] has yet to be fully resolved, but through the combined use of ultrastructural, immunolocalization, and flow cytometry analyses our evidence favors HSPA2 occupying an intracellular location and thus playing an indirect role in mediating sperm-egg recognition.[26] Specifically, we posit that HSPA2 facilitates the assembly and/or presentation of zona recognition complexes on the sperm surface.[25,26,72]

Support for this hypothesis rests with our demonstration that HSPA2 stably interacts with a number of multimeric complexes, with aggregate molecular weights of greater than 150 kDa, in spermatozoa from fertile normozoospermic individuals.[26] In proof-of-concept studies we have established that one of the major HSPA2 complexes harbors two additional proteins, sperm adhesion molecule 1 (SPAM1) and arylsulfatase A (ARSA), both of which have been implicated in interactions with the cumulus-oocyte complex.[73–79] Interestingly, this complex undergoes a marked, capacitation-associated translocation leading to the repositioning of ARSA to the outer leaflet of the sperm surface, a location compatible with a role in the mediation of sperm-ZP

interactions. Conversely, SPAM1 appears to reorient away from the sperm surface, possibly reflecting its primary role in cumulus matrix dispersal preceding sperm-ZP recognition.[25,26] In addition to aligning perfectly with the functional requirements of spermatozoa engaged in the process of fertilization, this regulated shift in surface expression is commensurate with the observation that spermatozoa in the advanced stages of capacitation lose their ability to bind hyaluronic acid.[80]

In recent unpublished studies, we have shown that the dynamic, capacitation-associated translocation of proteins appears to extend to additional HSPA2 client proteins thus raising the possibility that the chaperone holds a key role in priming the sperm surface architecture in advance of their interaction with the cumulus-oocyte complex. Such activity, in turn, appears to be driven by the tyrosine phosphorylation of HSPA2 during the latter stages of capacitation and can be completely abolished by incubation of spermatozoa in broad spectrum tyrosine kinase inhibitors.[25] Taken together, these results offer a rational explanation for why HSPA2 expression, hyaluronic acid binding and sperm-zona interaction are functionally linked and why they are all associated with male infertility; without HSPA2, neither the hyaluronidase receptor, nor the zona receptor(s), would be expressed in the coordinated manner needed to achieve fertilization.

MECHANISMS UNDERPINNING THE LOSS OF HEAT SHOCK PROTEIN A2 FROM THE SPERMATOZOA OF INFERTILE PATIENTS

A major goal of our ongoing investigations has been to determine how the incorporation of HSPA2 into the differentiating gamete becomes so dramatically disrupted in cases of infertility. Among the various possibilities that could account for the selective loss of HSPA2, genetic mutations in the encoding *Hspa2* gene, epigenetic regulation of *Hspa2* gene expression, and/or perturbations in protein expression/stability arising from exposure of developing germ cells to oxidative stress are currently under consideration. While the former explanation cannot be entirely ruled out, it appears to contradict evidence from transgenic mouse models in which the targeted ablation of the *Hspa2* gene leads to complete spermatogenic arrest (**see Section "THE ROLE OF HEAT SHOCK PROTEIN A2 IN MOUSE SPERMATOZOA"**). Similarly, although there is evidence that the methylation status of the *Hspa2* gene correlates with its transcription level in human somatic cells, these findings are not without controversy (reviewed by[71]). In contrast, oxidative stress is well known to play a fundamental role in the etiology of male infertility by negatively affecting sperm quality and function.[81]

As outlined previously, it is known that the HSPA2 protein is translated from an early stage in spermatogenesis and serves multiple functions being both necessary for the progression of meiosis and a major marker for the quality of spermiogenesis.[60] A defining characteristic of spermiogenesis is that it is extremely sensitive to oxidative damage owing to the fact that it is driven by the differential translation of proteins from long-lived mRNA species. While attention is usually focused on the damage that free radicals can inflict on DNA, RNA is equally vulnerable to oxidative attack, as is the process of protein translation.[82] In this light, the round spermatid may be uniquely vulnerable to oxidative attack since this cell type is replete with the mRNA species it will need to build a spermatozoon and is responsible for carefully orchestrating the movement of these mRNAs from ribonucleoprotein particles (RNP) to polysomes to affect their differential translation.[83] It is therefore possible that the reason HSPA2 expression is reduced in defective spermatozoa lacking the ability to bind to the ZP is that either the mRNA for this chaperone or the mechanisms for its translation have been oxidatively damaged during spermiogenesis.

An attack on mRNA integrity is suggested by previous studies revealing low levels of *Hspa2* mRNA expression in the defective spermatozoa of patients exhibiting oligoteratozoospermia or oligozoospermia associated with varicocele.[61,84] A particular role for oxidative stress is supported by the strong positive correlations that have been established between defects in sperm binding to hyaluronic acid polymers (mediated in part by SPAM1, one of the binding partners for HSPA2) and increased levels of peroxidative damage to the sperm plasma membrane.[85,86] The possibility that such an oxidative attack would strike late in spermatogenesis, when sperm differentiation is occurring, is suggested by a highly significant under-representation of phosphoglycerate kinase (PGK), mirroring the loss of HSPA2, in spermatozoa exhibiting an inability to bind to the ZP.[26] The significance of this finding is that the PGK isoform in spermatozoa (PGK2) is transcribed late in spermatogenesis to become the major PGK in spermatozoa.[87] The loss of this protein is therefore consistent with the disruption of protein translation during spermiogenesis, as the PGK2 mRNA migrates from RNP to polysomes, because of oxidative damage to the mRNA and/or disruption of the translation machinery itself. If this is the case then a number of other mRNA species that show the same orchestrated movement from RNPs to polysomes during spermatogenesis, should also be affected.[88] The assessment of the mRNA profiles of spermatozoa exhibiting a failure of sperm-egg interaction could, therefore, prove to be a valuable tool in evaluating sperm reproductive capacity and functional competence in infertile men.

Finally, as an alternative to mRNA damage, it is also possible that the lack of HSPA2 seen in spermatozoa of infertile patients may arise from a mechanism involving the targeted destruction of the protein itself. Consistent with this notion, recent work from our laboratory has shown that electrophilic aldehydes, such as 4-hydroxynonenal (4HNE), generated as a result of reactive oxygen species-induced lipid peroxidation are readily capable of adducting proteins localized within the head of human spermatozoa.[89] Furthermore, peptides belonging to HSP70 family members have been identified among the major 4HNE alkylated targets in these damaged cells.[89] While the primary impact of 4HNE covalently binding to proteins in mature spermatozoa is likely to involve conformational changes and/or aggregation leading to disruption of their functionality,[90] it may have a more pronounced effect in developing germ cells (**Figure 2**). Indeed in tissues such as the testis, which possess an intrinsic ubiquitin-proteasome system, such insults commonly lead to the activation of a protein degradation cascade that selectively eliminates damaged proteins in an effort to mitigate the impact of oxidative injury.[91–93] Alternative, ubiquitin-dependent lysosomal degradation mechanisms have also recently been reported for 4HNE-modified proteins.[94] Whether such mechanisms underpin the loss of susceptible proteins such as HSPA2 from maturing spermatozoa remains to be established. Similarly, it also has yet to be investigated whether aberrant expression of BAT3 might contribute to this phenotype through accelerated degradation of HSPA2[54] during human spermatogenesis.

CONCLUSIONS

An idiopathic failure of sperm-egg recognition ranks among the major reproductive lesions experienced in male infertility patients. Data from a number of independent laboratories suggest that this process is commonly impaired because of an underrepresentation of a key molecular chaperone, HSPA2, in pathologically defective spermatozoa. These findings accord with the view that molecular chaperones are critically involved in conferring upon spermatozoa the

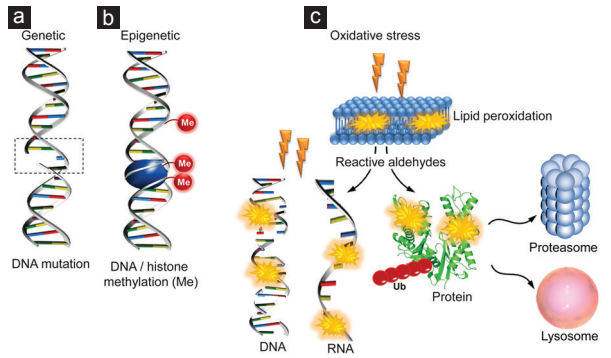

Figure 2: Mechanisms underpinning the loss of heat shock protein A2 (HSPA2) from the spermatozoa of infertile patients. Although the mechanism(s) underpinning the selective loss of HSPA2 from the differentiating gametes of infertile patients is currently unknown, several possibilities have been raised. These include: (**a**) genetic mutations in the encoding *Hspa2* gene, (**b**) epigenetic regulation of *Hspa2* gene expression, and/or (**c**) perturbations in protein expression/stability arising from exposure of developing germ cells to oxidative stress. With regard to the latter it is possible the oxidative attack could act directly to damage the *Hspa2* gene or mRNA transcript. Alternatively, the HSPA2 protein itself may be targeted for destruction following adduction by electrophilic aldehydes generated as a result of reactive oxygen species-induced lipid peroxidation. Such insults are known to lead ubiquitin (Ub)-dependent degradation via proteasomal or lysosomal pathways.

potential to interact with the oocyte during the sequential phases of sperm maturation.[22,95] These studies open up new research questions concerning the incidence of HSPA2 insufficiency in the patient population, the pathways by which this chaperone is incorporated into the differentiating gamete, how such incorporation becomes so dramatically disrupted in cases of infertility and the mechanisms by which HSPA2 regulates the differential surface expression of molecules involved in recognition of the oocyte-cumulus complex. Addressing these questions will have important implications for the diagnosis, treatment and prevention of infertility, and, in so doing, answer the long-standing call for evidence-based medicine in andrological practice.

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