ROLE OF UBIQUITIN LIGASE HUWE1 IN SPERMATOGENESIS

Kai Sheng

Experimental Medicine

McGill University, Montreal

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CONTENTS

ABSTRACT

During spermiogenesis, the replacement of histones initially by transition proteins and subsequently by protamines is vital for normal sperm formation. It is well known that this process requires proper function of the ubiquitin-proteasome system. We previously discovered a 480 kDa ubiquitin ligase named Huwe1 from testis and identified its ubiquitination activity towards all 4 core histones in vitro. We hypothesized that during spermiogenesis, Huwe1-dependent histone ubiquitination is responsible for its turnover. To test our hypothesis, we generated Huwe1 testis specific knockout males by crossing conditional Huwe1 knockout female mice (Huwe1$^{flox/flox}$) with hemizygous Ddx4-Cre males that express Cre recombinase in gonocytes just prior to birth and therefore prior to exit from quiescence and the establishment of the spermatogonia stem cell (SSC) pool. The Ddx4-knockout mice were totally infertile. Histological analysis unveiled that the spermatogenesis of Ddx4-KO mice did not reach remodeling phase suggesting that Huwe1 might play important roles in gonocyte transition or the mitotic phase of spermatogenesis. So far we have demonstrated that Huwe1 is required for the establishment and maintenance of spermatogonial stem cells (SSC). My part of the work from the present study was to explore the underlying mechanisms: 1) Silencing of Huwe1 in the C18-4 type A spermatogonia cell line resulted in a 30-40% decrease in proliferation rates. The cell number decrease was associated with a 30-40% down regulation of FoxO1 at the protein level, a transcription factor previously identified as necessary for SSC establishment. Instead of targeting FoxO1 for proteasome degradation, Huwe1 shows a protective effect toward FoxO1. However, decrease FoxO1 is not a major cause of this phenotype as overexpression of FoxO1 upon Huwe1 silencing failed to reverse the cell number loss. 2) As a hall marker of DSBs, an increased number of γH2AX intensive staining foci was found to co-localize with a germ cell marker in KO postnatal testis sections beginning at dpp5. Silencing of

Huwe1 in C18-4 cells followed by induction of DNA damage with hydroxyurea resulted in prolonged expression of gH2Ax consistent with increased gH2Ax staining seen in the KO testis. Assessment of some major regulators of the DNA damage repair pathway including RNF8, RNF168, NBS1, and ATM was performed on long terme culture SSC model with or without Huwe1 deletion. No significant change of those regulators was observed, suggesting loss of Huwe1 might act upstream of the DDR pathway. Future work should be directed at elucidating the molecular mechanisms by which Huwe1 regulates the DNA damage response.

RÉSUMÉ

Durant la spermatogénèse, les histones sont initialement remplacés par les protéines de transition et par la suite remplacés par les protamines. Ce processus est crucial pour le développement normal des spermatozoïdes. Il a déjà été démontré que ce processus requiert le bon fonctionnement du système ubiquitine-protéasome. Nous avons précédemment découvert une enzyme de ligation à l'ubiquitine de 480kDa, Huwe1, à partir d'extrait de testicule et nous avons identifié in vitro son activité d'ubiquitination envers les 4 principaux histones. Nous avons donc avancé l'hypothèse que, durant la spermatogénèse, l'ubiquitination des histones, dépendante de Huwe1, serait responsable de leur turn-over. Afin de tester notre hypothèse, nous avons généré des souris dont le gène Huwe1 est inactivé spécifiquement dans les testicules en croisant des femelles dont le gène Huwe1 est conditionnellement inactivé (Huwe1flox/flox) avec des males hémizygotes Ddx-4 Cre. Ces derniers expriment le gène Cre-recombinase dans les gonocytes juste avant la naissance et donc tout juste avant la sortie de quiescence et l'établissement des cellules souches spermatogoniales (SSC). Les souris knockout Ddx4 sont complètement stériles. Des analyses histologiques ont dévoilé que la spermatogénèse chez les souris KO Ddx4 n'a jamais atteint la phase de remodelage suggérant un rôle important pour Huwe1 dans les stades précoces de la spermatogénèse. Jusqu'à maintenant, nous avons démontré que Huwe1 est nécessaire pour l'établissement et le maintient des cellules souches spermatogoniales (SSC). Mon rôle dans cette présente étude était d'explorer les mécanismes à la base de ces observations: 1) Le silençage de Huwe1 dans des cellules spermatogoniales de type A a provoqué une diminution du taux de prolifération de 30 à 40%. Cette diminution du nombre cellulaire est associé avec une baisse de 30 à 40% des niveaux protéiques de FoxO1, un facteur transcriptionnel ayant été identifié précédemment comme un facteur nécessaire à l'établissement des SSC. Au lieu de cibler FoxO1

pour la dégradation par le protéasome, Huwe1 semble plutôt avoir un effet protecteur envers FoxO1. Pourtant, ce phénotype ne peut être attibuable à la perte de FoxO1 puisque la surexpression de FoxO1 dans le contexte où Huwe1 est silençé, n'a pas réussi à renverser le phénotype de perte cellulaire. 2) Nous avons observé une augmentation du nombre de foci marqués intensivement pour γH2AX, typique marquage des DSBs, avec un marqueur de cellule germinale dans des testicules KO postnatale débutant à dpp5. Le silençage de Huwe1 dans les cellules C18-4 suivies par induction de lésions de l'ADN avec de l'hydroxyurée a donné lieu à l'expression prolongée de γH2AX compatible avec une coloration accrue γH2AX vu dans le testicule knockout. Une évaluation de quelques principaux régulateurs de la voie de réparation de dommage d'ADN (DDR) incluant RNF8, RNF168, NBS1 et ATM a été réalisée sur des cultures SSC avec ou sans délétion de Huwe1. Les travaux futurs devraient viser à élucider les mécanismes moléculaires par lesquels Huwe1 réglemente la réponse aux dommages de l'ADN.

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ABBREVIATIONS

4OHT: 4-hydroxytamoxifen

A_s: Spermatogonia A_{Single}

A_{pr}: Spermatogonia A_{paired}

A_{al}: Spermatogonia $A_{aligned}$

AR: androgen receptor

ATM： Ataxia telangiectasia mutated

ATR: ataxia telangiectasia and Rad3-related protein

cAMP: cyclic AMP (adenosine monophosphate)

CHK1: Checkpoint kinase 1

CHK2: Checkpoint kinase 2

CDC25A: cell division cycle 25 homolog A

CDK1: Cyclin-dependent kinase 1

CCND1: Cyclin D 1

CCND2: Cyclin D 2

DAG: 1,2-diacylglycerol

DBD: DNA binding domain

DES: Diethylstilbestrol

DNA: deoxyribonucleic acid

dpc: Days post coitum

dpp: days post partum

ER: estrogen receptor

FBS: Foetal bovine serum

FSH: Follicle-stimulating hormone

GDNF: Glial cell derived neurotrophic factor

GTPase: enzyme to hydrolyze guanosine triphosphate

HUWE1: HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase

HGF: hepatocyte growth factor

HSP: Heat shock protein

ICC: Immunocytochemistry

IHC: Immunohistochemistry

IGF-1: Insulin-like growth factor 1

IGF-1R: Insulin-like growth factor 1 receptor

IP3: inositol triphosphate

kDa: Kilodalton

LBD: ligand binding domain

MKP: MAPK phosphatase

MRN: Mre11-Rad50-Nbs1 complex

MDC1: Mediator of DNA damage checkpoint protein 1

Mcl-1: Induced myeloid leukemia cell differentiation protein 1

NES: Nuclear export signal

NLS: Nuclear localization signal,

NBS1: Nibrin

NOA: non-obstructive azoospermia

PDGF: Platelet-derived growth factor

PDGFR: PDGF receptor

PDPN: Podoplanin

PGC: Primordial germ cell

PI3K: phosphatidylinositol 3-kinase

TP53BP1: Tumor suppressor p53-binding protein 1

P21: $p21^{Cip1}$/cyclin-dependent kinase inhibitor 1

P16: cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1

P27: $p27^{Kip1}$/Cyclin-dependent kinase inhibitor 1B

RA: Retinoic acid

ROS: reactive oxygen species

RNF8: Ring finger protein 8

RNF168: Ring finger protein 168

SCF: Stem cell factor

SSC: Spermatogonial stem cell

VEGF: vascular endothelial growth factor

TGF: transforming growth factor

CHAPTER 1 INTRODUCTION

1.1 Ubiquitination

1.1.1 Discovery and background

Proteins are the most abundant organic compounds present in all living organisms. They consist of chains of amino acid residues and so are also called polypeptides. Proteins differ from one another in sequence and therefore molecular size, three-dimensional structure, and function. So far, nearly 100,000 proteins derived from 25,000 genes have been identified from the human body. They are responsible for nearly every task of cellular life: some of them are involved in cell structural support, movement, storage, some of them are hormones and others have different enzymatic activities[1,2].

Nearly a century after the initial discovery of proteins in the 19th century by Jacob Berzelius, scientists had immersed themselves to study protein synthesis and to determine their three-dimensional structures as well as analyze their functions. However, how proteins are degraded had seldom been asked and studied. In 1942, Schoenheimer provided scientific evidence in support of a precise balance of turnover and synthesis. The next important step of discovery in protein degradation was in 1953 when Simpson measured the release of amino acids from cultured liver slices and found that this process was energy-dependent [3]. In 1978, Hersko and his colleagues resolved a heat-stable polypeptide required for the activity of an ATP-dependent proteolytic system from reticulocytes. This polypeptide was subsequently identified as ubiquitin, a 76-amino-acid, highly conserved protein present in all eukaryotes[4,5].

By using this extracted peptide, Aaron Ciechanover [6], Avram Hershko [7] and Irwin Rose made a series of ground breaking discoveries in the late 1970s and early 1980s [6,8,9]. They depicted precisely a 3-step enzymatic cascade by which proteins were tagged by the polypeptide ubiquitin and subsequently degraded in the 26S proteasome [10-12]. The Nobel Prize in Chemistry for 2004 was shared by these three scientists for these fundamental discoveries[5].

1.1.2 The ubiquitin conjugation cascade

Ubiquitination is carried out by a three-step cascade of enzymatic reactions which involves three types of enzymes, known as E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) [13] (Figure 1). In the first step, E1 activates ubiquitin by forming a high energy thioester linkage between its active site cysteine and the carboxyl group of the terminal residue (G76) of the ubiquitin, through an ATP-dependent fashion. Next, the activated ubiquitin is transiently transferred to E2. In the last step, E3 recognizes the target protein substrate and binds both the substrate and the E2 that is thioesterified with ubiquitin. Concomitantly, ubiquitin is transferred from the E2 to the ε-amino group of a lysine residue of the substrate (Figure 2). Given the function of target recognition, E3s render the specificity to the ubiquitination[13]. The discovery of approximately 600 genes that encode E3s in the human genome supported this functional specificity. Compared with the enormous number of E3s, the human genome contains only two E1s and approximately 30 E2s[14].

1.1.3 Types of ubiquitination and function

Two types of ubiquitination exist, known as monoubiquitination and polyubiquitination. Monoubiquitination is described as the addition of one ubiquitin molecule to one or more lysine

residues of a substrate protein whereas polyubiquitination involves conjugation of chains of ubiquitin to the substrate. The polyubiquitin chain is made by adding additional ubiquitin moieties to one of the lysine residues of the former one. Notably, ubiquitin has 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63) on which an additional ubiquitin can be conjugated[15]. Monoubiquitination and polyubiquitination can co-exist on different lysine residues of the protein substrate[16]. The possible combinations of mono ubiquitination as well as various types of polyubiquitination therefore produce an extremely diverse code, by which countless functional potentialities are rendered[17].

The canonical degradative function of ubiquitination is classically mediated by K48 (Lysine 48)-linked polyubiquitin chains. In this ubiquitin proteasome pathway (UPP), K48–linked polyubiquitin tagged proteins are recognized by the 26S proteasome complex and undergo degradation. The 26S proteasome consists of two subcomplexes: a 20S catalytic core and 19S cap particle. The 20S unit is bound at one or both ends by a 19S unit and forms the tube shape of the 26S proteasome [12,18]. The UPP is considered to be the major mechanism responsible for protein turnover in eukaryotic cells [19-22]. Another well-studied polyubiquitin chain is the K63 (Lysine 63)-linked chain which is not associated with proteasomal degradation [23]. Instead, this chain orchestrates other cellular processes such as endocytic trafficking, inflammation, translation, and DNA repair[18]. More recently, six 'atypical' chain types (linked via Lys6, Lys11, Lys27, Lys29, Lys33 or Met1) have also been identified yet the functions of such chains remain largely unknown[24-27]. On the other hand, the cellular functions of monoubiquitination include membrane trafficking, endocytosis viral budding and chromatin remodeling. The alteration of chromatin structure affects the accessibility of transcription factors to genomic sequence and therefore regulates gene transcription. Monoubiquitination of histones is also able to modulate transcription

through other mechanisms: by either serving as binding docks for some transcription factors (activate or inhibit) or to further trigger other post-translational modifications of histones (methylation, acetylation). Recent studies have provided evidence showing that monoubiquitination and polyubiquitination cooperate with each other. For example, in the DNA damage response pathway, the formation of a monoubiquitination of H2A/H2AX is a prerequisite for its K63-linked polyubiquitination on K13-15.

1.1.4 E3 ubiquitin ligase

There are two major types of E3s in eukaryotes: HECT (homologous to E6-AP C-terminal) type and RING (really interesting new gene) type, defined by the homology of their E2 binding domain: either a HECT or a RING domain. Among over 600 E3s in the human genome, most (~95%) belong to the RING family and only 28 belong to the HECT family[28-30]. Both RING and HECT E3s remain largely uncharacterized, and functional data are available for only a small minority of them[31].

1.1.4.1 RING type E3

The RING domain was originally discovered by Freemont in 1993 and described as a unique three-dimensional structure known as a "cross-brace". It usually contains 40–80 amino acid residues with eight highly conserved cysteine and histidine residues that help maintain the overall structure through binding two atoms of zinc [28]. The canonical consensus sequence of these amino acids is $C\text{-}X_2\text{-}C\text{-}X_{(9\text{-}39)}\text{-}C\text{-}X_{(1\text{-}3)}\text{-}H\text{-}X_{(2\text{-}3)}$ - $C/H\text{-}X_2\text{-}C\text{-}X_{(4\text{-}48)}\text{-}C\text{-}X_2\text{-}C$ (X is any amino acid). Depending on the presence of C or H in the fifth conserved positions, RING type ligases can be classified into

three sub-groups: C3HC4 (also known as RING-HC/HC-type RING), C3H2C3 (also known as H2-type RING/RING-H2) and C4HC3 (also known as CH-type RING/RING-CH) fingers [28].

The RING domain doesn't have intrinsic catalytic activity in the last step of ubiquitination. It is now widely accepted that it functions in binding and activating the E2 enzyme to mediate the ubiquitin transfer [32]. The first type of RING type E3 ligase was one in which the RING finger containing protein is part of multi-subunit protein complexes such as SCF (Skp1-cullin-F-box), APC (anaphase promoting complex) and CBC (cullin-elongin B and C) ligases. Taking SCF complex as an example, it consists of the bridging protein Skp1 which connects backbone and F-box, the backbone Cullin1 (Cdc53 in yeast), a RING finger protein ROC1 (or Rbx1) and an F-box protein. The F-box protein varies from one SCF complex to another, for example, SCF^{Cdc4}, $SCF^{\beta\text{-}TrCP}$ and renders the substrate specificity of the SCF complex [33,34]. Some RING type ligases have their substrate binding site and the catalytic RING domain on the same polypeptide and have therefore been termed single-subunit RING E3 ligases, such as Mdm2, c-Cbl, BRCA1. Single-subunit RING E3 ligases can also be divided into three subgroups based on the type and position of associated domains: tripartite motif (TRIM) containing, PA (protease-associated domain)-TM (transmembrane domain)-RING E3s, RING between RINGs (RBR) and membrane-associated RING-CH (MARCH) families [28].

1.1.4.2 HECT type E3

The HECT domain was originally discovered in the E6-associated protein (E6-AP). It contains approximately 350 amino acids and is located on the C-terminal end of HECT family ligases. Unlike the RING domain which functions as a scaffold that brings E2 and substrate together, the HECT E3 possesses intrinsic catalytic activity in this bilobed HECT domain. The C lobe has a

conserved cysteine residue which is required for ubiquitin-thiolester formation whereas the N lobe binds the E2 enzyme. The overall structure of HECT domains from the HECT family are similar, but the orientation and position of the C lobe and N lobe varies from one to another. The substrate binding specificity of HECT ligase is determined by one or more protein-protein interaction domains located N terminal to the HECT domain. Three sub-families exist according to the architecture of the N terminus: The Nedd4 family (9 members), the HERC family (6 members) and the other HECTs (13 members) [35]. Nedd4 family ligases are also known as C2-WW-HECT ligases as the N terminal of Nedd4 family ligases contain a C2 domain and two or four WW domains [36]. The C2 domain binds Ca2+ and phospholipids and is involved in targeting the HECT E3s to intracellular membranes. The WW domain mediates ligase-substrate association through interactions with a variety of proline-rich motifs and proline-containing phosphorserine/threonine sequences of the protein substrate. C2-WW-HECT E3s typically regulate the endocytosis and trafficking of plasma membrane proteins by altering the stability of both transmembrane receptors and intracellular substrates [37]. The HERC ligases possess regulator of chromosome condensation 1 (RCC1)-like domains (RLDs) at the N-terminal [38]. The RLD has a seven-bladed β-propeller fold, in which one side of the propeller interacts with chromatin and the other might be a guanine nucleotide-exchange factor (GEF) for the small GTPase Ran [30,39].

1.2. UPS DURING SSC ESTABLISHMENT AND MAINTENANCE

1.2.1 Overview of Rodent Spermatogenesis

Sperm have a vital role in the continuity of a species by not only contributing half of the genetic information to the next generation but also by possessing the ability of reaching the egg in the female reproductive ducts. This process of generation of spermatozoa is referred to as

spermatogenesis. It is a complex developmental process that encompasses multiple molecular mechanisms but occurs as an orderly spermatogenic cycle. The spermatogenic cycle has been well defined based on morphology of individual germ cells and their grouping in cellular associations [40-42]. For example, mouse spermatogenesis can be divided into 12 sequential stages, while rat has 14 stages. Spermatogenesis is driven by the Spermatogonia Stem Cells (SSC) that arise from gonocytes in the postnatal testis, which arise from primordial germ cells (PGCs) during fetal development. PGCs are a small cluster of alkaline phosphatase-positive cells in the epiblast stage embryo at about 7–7.25 days post coitum (dpc). PGC specification is dependent on the expression of BMP4 and BMP8b from the extraembryonic ectoderm. The PGCs stay proliferative until the formation of the allantois. During the formation of the allantois, about 3000 PGCs migrate to and colonize the genital ridges [43]. Once they become resident in the gonadal primordium, they are referred as gonocytes. At about 13.5 dpc, the fetal gonocyte undergoes mitotic cycles followed by a quiescent period during which intensive DNA methylation takes place. Then shortly after birth (PND 3-5), the neonatal gonocyte migrates from the center of the newly formed seminiferous core to the basement membrane. Concomitantly, they re-enter mitosis and generate both the differentiating spermatogonia lineage and the undifferentiating spermatogonia lineage that includes spermatogonia stem cells SSC [44,45].

Thereafter, spermatogenesis can be divided into three phases, the mitotic phase (also known as proliferative phase), the meiotic phase, and the remodeling phase (also known as spermiogenesis) [41,46,47] . In the mitotic phase, the undifferentiating spermatogonia are regulated importantly by platelet-derived growth factor (PDGF) and 17β-estradiol, while the differentiating spermatogonia are induced by retinoic acid (RA) [47]. The differentiated lineage then undergoes two rounds of

division which produces the haploid gametes from diploid spermatogonia. During the first meiotic division (meiosis I), chromosome pairs undergo homologous recombination through which genetic material is exchanged between maternal and paternal chromosomes. This process involves the formation of synaptonemal complexes in which double strand (ds) DNA breaks occur followed by repair. Following meiosis I, the secondary spermatocytes undergo another cell division (meiosis II) resulting in the separation of individual chromatid strands to generate two spermatid cells. In the final stage of spermatogenesis, spermiogenesis, major visible changes are observed as follows: the formation of the sperm tail which consists of a central 9+2 axoneme of microtubules and accessory proteins essential for flagellar movement; the formation of the acrosome from the Golgi apparatus and which contains digestive enzymes required for breaking down the outer membrane of the zona pellucida; the rearrangement of organelles such as mitochondria, centrioles; the shedding of the residual body (cytoplasm) and most importantly, the nucleus elongates and condenses. The nuclear condensation starts with the initial replacement of somatic histones by transition proteins and subsequently by sperm-specific protamines. Protamines render a higher order of DNA packaging comparing with that of histones in somatic cells. Such chromatin condensation is believed to protect the paternal genetic integrity while the spermatozoa travel through both the male and female reproductive ducts. Therefore, it is vital for normal fertilization. Yet, the spermatozoa are mature but lack motility so far. Sertoli cells release the non-motile spermatozoa into the lumen of the seminiferous tubule in a process called spermiation. They also secrete testicular fluid which facilitates the transport of spermatozoa to the epididymis along with the aid of peristaltic contraction. Finally, in the epididymis, the spermatozoa gain motility and become capable of fertilization [41,46,47].

1.2.2 Spermatogonial Stem Cell (SSC)

Spermatogenesis is driven by the Spermatogonia Stem Cells (SSC) which represent a small subpopulation of undifferentiated spermatogonia. The undifferentiated spermatogonia consist of type A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) subtypes. Among them, A_s spermatogonia have been widely accepted as the SSC population which possesses the unique self-renewal ability [47]. Upon division, A_s cells can produce two identical A_s. Incomplete cytokinesis leads to an intercellular bridge connecting two A_s thereby forming A_{pr}. A_{pr} then undergo a series of mitotic divisions with incomplete cytokinesis to form interconnected cohorts of 4, 8, 16, and sometimes 32 cells that constitute the A_{al} spermatogonia [40,47](Figure 4). Upon retinoic acid (RA) signaling, the majority of A_{pr} and A_{al} will stop cell division and start to differentiate. As the ground state of spermatogenesis, impaired function of SSC leads either to a depletion of the SSC pool or causes a block in initiation of differentiation which consequently leads to diseases such as non-obstructive azoospermia (NOA). It affects approximately 1% of adult males and 10-15% of all male infertility cases. Conversely, overexpansion of SSC due to mis-regulation of self-renewal functions is thought to be a forerunner of testicular germ cell tumors (TGCTs) [47].

To understand the molecular mechanisms and cell-signalling pathways of SSC will help to provide therapeutics for curing male infertility as well as TGCTs. However, the studies on SSC has been hampered by two major difficulties. 1) The shortage of study material. This is because SSCs constitute only 0.02–0.03% of germ cells, which amounts to 2–3 $x10^4$ cells per mouse testis. 2) Lack of a specific marker. This makes it hard to distinguish SSC from extremely heterogeneous spermatogonial population. The impetus to SSC study has arrived along with the establishment of several techniques in the last two decades.

SSC transplantation was developed in 1994 by Brinster and colleagues [48]. Basically, germ cells are isolated from the testes of donor animals and transplanted into the testicular seminiferous of infertile recipients. Following microinjection into seminiferous tubules of testes, the transplanted SSC reinitiate spermatogenesis and produce functional sperm. As only a stem cell can produce and maintain a colony of spermatogenesis, each colony represents a single SSC. Therefore, the SSC transplantation technique provides a quantitative functional assay to characterize stem cell activity in any donor cell population [48].

Fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) has enabled investigators to characterize SSC based on cell surface markers. For FACS, basically, a fluorescent-conjugated antibody against the cell surface antigen is incubated with the heterogeneous testis cell suspension. The marker^{+} and marker^{-} populations are separated by flow cytometry and each fraction will be transplanted into infertile recipient mice to determine the relative stem cell activity. So far, mouse SSC is described as a6-Integrin (CD49f)$^{+}$, b1- Integrin (CD29)$^{+}$, THY-1 (CD90)$^{+}$, CD9^{+}, GFRa1^{+}, CDH1^{+}, av-Integrin (CD51)$^{-}$, c-KIT (CD117)$^{-}$, major histocompatibility complex class I (MHC-I)$^{-}$, CD45^{-} [49-53]. Using combinations of positive and negative markers, it is now possible to achieve significant enrichment (100- to 200-fold) of mouse SSC from the original pool.

During the last decade, several groups have reported successful culture of SSC from different mouse strains. SSC reside in the niche, which is comprised of somatic cells including Sertoli cells, Leydig cells, Myoid cells which produce numerous chemokines and growth factors. Therefore, the principle to maintain SSC culture in vitro is to mimic what the SSC niche provides in vivo. Three main factors are critical for survival and expansion of SSC in vitro culture. They are the basic medium, appropriate growth factors and feeder cells [54-56]. Basically, FACS/ MACS sorted germ

cell populations are cultured on STO or mouse embryonic fibroblasts (MEF) with a serum-free medium. By adding and removing different combinations of growth factors into the medium and observing whether this promotes or inhibits SSC, several key growth factors have been identified including GDNF (Glial cell-derived neurotrophic factor), LIF (Leukemia inhibitory factor), EGF (Epidermal growth factor), and FGF (Fibroblast growth factors) [55]. Successfully cultured SSC are able to proliferate for several months to years. The in vitro expansion of SSC during prolonged culture time provides a comparatively large quantity of study material for biochemical and molecular biological analyses. Loss- and gain-of-function study of a specific gene in SSC culture is able to be performed thanks to the development of knockdown and knockout techniques such shRNA and CRISPR/Cas9 system. Nowadays, the combination of in vitro manipulation of SSC and transplantation is the standard method for dissecting both extrinsic and intracellular molecular mechanisms of SSC.

1.2.3 Molecular mechanisms of SSC establishment and maintenance

At birth, gonocytes are in a quiescent state. They reside within the center of seminiferous cords. However, to form the SSC pool, gonocytes are required to migrate to the basement membrane and resume proliferative activity. It is believed that the transition is controlled by a sophisticated regulation that allows functional genes to turn on or off at the correct time. The detailed mechanisms still remain largely unknown. A few genes have been shown to be differentially expressed between gonocyte subpopulations. For example, Nanog decreases from being expressed in 13% of fetal mitotic gonocytes to 1% in quiescent gonocytes. GDNF receptor complex GFRa1/RET, is found in mitotic fetal but not in quiescent gonocytes [57]. Several other genes are associated with the gonocyte to SSC transition which normally happens between PND3-PND5 in

rodents. For example, activin βA levels appeared to decrease in parallel with an increase in its inhibitor, follistatin, during the transition from gonocytes to spermatogonia [58]. Meredith et al. showed that the subcellular localization of FoxO1 (Forkhead transcription factor 1) translocates from the cytoplasm to the nucleus during PND1 to PND3 [59]. Corresponding to its translocation is the activation of downstream target genes including Ret (Rearranged during transfection), Dgr4 (deoxyglucose resistant-4), Dppa4 (Developmental Pluripotency Associated 4). While the functional significance of this developmentally regulated shift in sub-cellular location is not clear yet, FoxO1 is regarded as a marker for the neonatal gonocyte to SSC transition [59].

Sertoli cells are thought to be the most important contributor to SSC self-renewal and maintenance as they are the only somatic cell type that directly interact with SSC and secrete growth factors and chemokines which control SSC self-renewal activities. It is believed that the Sertoli cell regulates SSC self-renewal capacity through at least four pathways [60] [46] (Figure 5). The most important one is the GDNF/RET/GFRA1 pathway. GDNF was the first identified obligatory niche factor for SSC maintenance and has been extensively studied. GDNF signaling acts via the RET tyrosine kinase present on undifferentiated type A-spermatogonia and which requires a ligand-specific co-receptor GFRA1. GDNF/RET/GFRA1 activates downstream intracellular signaling mechanisms including PI3K/AKT and SFK which influence SSC self-renewal transcription factors, such as B cell CLL/ lymphoma 6 member B (BCL6B), ETS variant 5 (ERM; also known as ETV5), DNA-binding protein 4 (ID4), LIM homeobox 1 (LHX1), BRACHYURY (T), and POU class 3 homeobox 1 (POU3F1) [54,61-64]. Another growth factor secreted by Sertoli cells and which is able to stimulate SSC self-renewal is FGF2. A recent study has demonstrated that FGF2 relies on MAP2K1 activation to drive SSC self-renewal via upregulation of ETV5, BCL6B, and LHX1genes. A subsequent study indicated that FGF2 might regulate mouse SSC proliferation and

stem cell activity in vitro via autocrine mediated phosphorylation of AKT and ERK1/2 pathway [65,66]. The third signaling pathway is CXCL12–CXCR4 signaling. CXCL12 encodes a chemokine that is expressed and secreted by the Sertoli cell and acts on SSC via its receptor CXCR4. Loss of function experiments of CXCL12 or CXCR4 have shown its importance in regulating SSC self-renewal. However, the downstream intracellular signaling pathways have not been defined [67,68]. Interestingly, GDNF and FGF2 regulate the expression of CXCR4 mRNA in $Thy1^+$ spermatogonial cell cultures, while disruption of CXCL12-CXCL4 signaling also leads to a decrease in FGF2 [68]. These studies suggest a cooperative network controlling SSC self-renewal which is comprised by CXCL12, FGF2, and GNDF signaling. Finally, CCL9 encodes a chemokine that facilitates Sertoli cell chemoattraction of undifferentiated type A-spermatogonia through its receptor CCR1. Both CXCL12/CXCR4 and CCL9/CCR1 signaling are regulated by ERM which is predominantly expressed within Sertoli cells [69].

Several transcription factors that are not influenced by the niche are also indispensable for SSC maintenance. One pair of these crucial regulators is PLZF (promyelocytic leukemia zinc finger) [70] and SALL4 (Sal-like protein 4) [71,72]. PLZF is expressed during mouse embryogenesis and into the postnatal testis (restricted to gonocytes and undifferentiated spermatogonia) and has been characterized as a DNA-binding transcriptional repressor of cell cycle progression [70-72]. Similarly, Sall4 is expressed in A_s A_{pr} and A_{al} spermatogonia and its expression pattern overlaps with PLZF. SALL4 is a zinc finger transcription factor which is involved in normal development, as well as tumorigenesis. Mice lacking PLZF undergo a progressive loss of spermatogonia with age caused by unrestricted exit from quiescence of spermatogonia, resulting in inappropriate activation of meiotic checkpoints and increased apoptosis. Expression of genes directly regulated by Plzf in other model systems such as Ccna2 and Myc were not altered in PLZF KD testis. However,

expression of several other groups of genes was perturbed, such as genes involved in metabolism (Gpd1, Cyp11a1, Hsd17b1, Ash2l), RNA binding (Rbm5, Rbm9, Paip1, Pabpc1), cell cycle control (Ccnd2, Ches1), cytoskeletal and cell-junction components (Knsl7, Col4a3bp, Cldn11) and transcription factors (Dmrt2). Hobbs et al. showed that Sall4 and Plzf mutually antagonize each other [73]. Specifically, Plzf antagonizes Sall4 function by displacing Sall4 from cognate chromatin to induce Sall1 expression. In turn, during SSC differentiation, Sall4 sequesters Plzf to pericentric heterochromatin, to induce expression of Kit which is required for differentiation [73]. Even though the expression of Plzf doesn't respond to GNDF signaling, it has been reported to regulate GDNF signaling by inducing transcription of the mTORC1 inhibitor REDD1 [74]. It is known that the expression of GDNF receptor complex RET and GFRa1 subunits is suppressed by the activity of mTORC1. Thus, spermatogonia from Plzf null mice possess elevated mTORC1 activity which attenuates expression of RET and GFRa1 thereby inducing a greater propensity for apoptosis and promoting premature differentiation.

1.2.4 UPS in regulation of SSC function

As shown above, previous studies of regulation of SSC function focused mainly on the influence of transcription factors which allows genes to spatial-temporally turn on/off. In addition to the transcriptional regulation network, there is also expected to be a regulatory layer at the protein level which stabilizes useful proteins and turns over unwanted ones. As the major protein turnover mechanism in somatic cells, the UPS has been implicated in the control of mammalian gametogenesis through regulating protein stability. Several ubiquitin-related genes specific to gonocytes and spermatogonia have been identified including UBC2, UBC4, UBR5, Huwe1, UCH-

L1 and FBXW7[75-77]. Their expressions is tightly regulated during testicular development, suggesting roles during spermatogenesis. Yet, very few detailed regulatory functions of these ligases are known so far.

Ubiquitin ligase FBXW7 (F-box and WD-40 domain protein 7) has been identified as a negative regulator of SSC self-renewal in 2014 by Takashi Shinohara[76]. FBXW7 is a component of the SCF-type (Skp1-Cullin-F-box–type) ubiquitin ligase complex, and functions in target recognition. The expression of FBXW7 is restricted to undifferentiated spermatogonia. Fbxw7 mRNA expression in testis has no response to self-renewal factors, including FGF2 and GDNF. It thought to be controlled by negative regulators such as Zbtb16 and positive regulators Id2/Id3/Id4 in a cell cycle-dependent manner [78]. They also showed by SSC transplantation that Fbxw7 overexpression compromises SSC activity. Conversely, Fbxw7 deficiency increased proliferation of undifferentiated spermatogonia and enhanced SSC colonization. FBXW7 targets in SSC include the oncogene myelocytomatosis (MYC) and cyclin E1 (CCNE1) which are upregulated in FBXW7 deficiency mice. Transplantation of SSC depleted of Myc/Mycn or Ccne1/Ccne2 showed a compromised SSC activity, while SSC that overexpress Myc, but not Ccne1, increased the colonization. Collectively, FBXW7 negatively regulates SSC self-renewal by counteracting positive regulators such as MYC and CCNE1.

βTrCP is one of the most highly studied and best characterized ubiquitin ligases in cancer cells and plays important roles in regulating cell cycle and apoptosis, but its function in SSC was only revealed in 2009 [79]. βTrCP is the substrate recognition subunit of a SCF ubiquitin ligase. There are two isoform in mammals BTRC and FBXW11 (also known as βTrCP1 and βTrCP2). Both of them have been detected in spermatogonia yet they show nonredundant roles in spermatogenesis. Gene inactivation of βTrCP1 (KO1) affects metaphase 1 spermatocytes with no effect on

spermatogonial development. However, βTrCP2 deficiency mice (KD2) or hybrid strain (KO1/KD2) with insufficiency of both isoforms results in disrupted organization of germ cells [80]. KO1/KD2 testes show disordered tissue architecture and spermatogonial stem cell dislocation at the lumen with loss of the tight junction marker E-cadherin. Snail1 is a substrate of βTrCP and is responsible for the phenotype. As a transcriptional repressor of E-cadherin, Snail1 showed significant upregulation in KO1/KD2 mice resulting in decreased E-cadherin level. Silencing Snail1 in the βTrCP deficient testis was able to restore the expression of E-cadherin and reverse the impairment in cell–cell interaction in spermatogenesis [79].

Other lines of evidence of UPS regulating spermatogonia development include: the study that found that the deubiquitinating enzyme Uchl1 is associated with both symmetrical and asymmetrical division to maintain the stem cell pool and differentiation of progeny, yet the direct substrate is still unknown [81,82]; the finding by Spencer at 2013 showed that PLZF stability is affected by PLZF phosphorylation rendering it prone to ubiquitylation directed degradation, yet the ligase responsible for PLZF is also still unknown [83].

A major difficulty in studying ligase regulatory functions on SSC is to identify its substrates. The straightforward method of substrate identification requires immunoprecipitation of ligase–substrate complexes followed by MS. However, the interaction of ligase–substrate is generally too weak and transient. One approach that has been recently developed in order to address this gap is affinity-based proteomics strategies [84]. Basically, upon trypsinolysis the Lys-ϵ-Gly-Gly (diGly isopeptide bond) fragment is formed from ubiquitinated proteins. Pulling down all peptides containing diGly motif using antibodies recognizing the diGly motif followed by MS will provide the global quantitation and identification of ubiquitinated proteins. Comparison of global proteomic differences between the wildtype and cell that has loss function of certain E3 ligases

will give us more clues of the ligase substrate [85] [86]. However, this method still requires large quantities of proteins and so may not be able to be performed on SSC. Currently study of substrates of E3 ligase on SSC relies on screening known substrates which have been identified in other systems. Therefore, study of the UPS in SSC still requires new technology or strategies to be developed.

1.3. STUDY OBJECTIVES

1.3.1 The protein of interest: Huwe1

Huwe1 is a 480 kDa large ubiquitin ligase that belongs to the SI(ngle)-HECT E3s subfamily. The HECT domain is located in the C terminus of HUWE1 similarly to other HECT type ligases. In the N terminus, there are two DUFs (Domain of unknown function). In the middle, there are three domains associated with ubiquitin binding activity. They are UBA (ubiquitin-associated domain). UBA is known to bind both mono- or poly-ubiquitin, and WWE domain which is predicted to mediate specific protein-protein interactions in ubiquitin and ADP ribose conjugation systems. However, the precise roles of these domains still remains to be defined. Huwe1 also has a conserved BH3 domain next to the WWE domain through which it interacts with the Bcl-2 family member Mcl1. The middle portion also contains an NLS (Figure 6) [87-90].

Huwe1 was first reported in 2005 by four different research groups including ourselves. These studies identified four different substrates: histones [90], Mcl-1 [89], c-Myc [87], and p53 [88]. Not surprisingly, Huwe1 was given different names such as ARF-BP1, HECTH9, MULE and E3^{histone}. Additional substrates of Huwe1 have been identified by recent research and include Cdc6 [91], N-Myc [92], HDAC2 [93], MyoD [94], Dishevelled [95] and BRCA1 [96]. Like many other E3 ligases with

multiple substrates, the precise substrate(s) degraded is cell type and cell condition dependent. Turnover of different substrates will have different consequences for multiple aspects of cell cycle control and cell fate determination. Three of its well-known substrates are, Mcl-1, MYC and p53 [39,88,89,97-100]. Mcl-1 is an antiapoptotic protein, MYC is an oncogene while p53 is a proapoptotic protein. Given the totally divergent functions of these substrates, Huwe1 mediated ubiquitination can result in either increased tumor cell survival or death. Under unstressed conditions, Huwe1 directly binds to and ubiquitylates p53. However, when facing DNA damage, Huwe1 preferably targets Mcl-1 as well as CDC6 for protein ubiquitination. Therefore, in the cancer research field, it is still controversial whether Huwe1 is a tumor suppressor or an oncogene.

In testis, Huwe1 expressed in germ cells prior to spermatogonia formation [75]. Huwe1 mRNA can be detected in rat gonocytes by qPCR at PND3. The transcript levels of Huwe1 gradually go down upon gonocyte development. The expression of Huwe1 in spermatogonia is only 50% comparing with gonocyte. Correspondingly to its transcription, inmmunostaining revealed that Huwe1 is highly expressed in the early phase of spermatogenesis. Meanwhile, we showed that Huwe1 is able to poly-ubiquitinate histones in vitro and we proposed that it plays a role in histone removal during chromatin condensation in elongating spermatids [77]. Interestingly, we noted that Huwe1 is expressed mainly in the nucleus of PND 3 gonocytes while in the cytoplasm of PND 8 spermatogonia. This translocation suggests its role in germ cell development [75].

1.3.2 The basis of the study

Previous work in our laboratory supports a role for ubiquitination during spermatogenesis. We found that the rate of ubiquitin conjugation increases during the first wave of spermatogenesis. Interestingly, induction of an E2 conjugating enzyme UBC4-testis correlates with the increase in

conjugation. UBC4-testis is an isoform of UBC4 which is highly expressed in round spermatids and early elongating spermatids [101]. Histones are known to be ubiquitinated and degraded in early elongating spermatids and appeared to be UBC4-dependant. Indeed, by using ^{125}I-labeled histone H2A as a substrate, and in presence of UBC4, we biochemically identified an E3 ubiquitin ligase. Mass spectrometry revealed that this ligase is identical with the E3 named Huwe1. We identified its ubiquitination activity towards all 4 core histones in vitro and showed that it was dependent on UBC4. We therefore hypothesized that during the remodeling phase (spermiogenesis), Huwe1-dependent histone ubiquitination is responsible for histones turnover [90].

1.3.2.1 The phenotype of Huwe1 germ cell specific knockout mice

To test our hypothesis, we generated Huwe1 germ cell specific knockout male (Huwe1 -/-) by crossing conditional Huwe1 knockout female mice (Huwe1$^{flox/flox}$) with hemizygous Ddx4-Cre males that express Cre recombinase before gonocytes exit the quiescent stage and start to establish the SSC pool. The adult knockout mice have a severe defect in spermatogenesis characterized by smaller testis, degeneration of seminiferous tubules, and absence of spermatozoa in the epididymis and consequently they were totaly infertile. To our surprise, histological analysis revealed that spermatogenesis in Ddx4-KO mice did not reach the remodeling phase indicating that Huwe1 plays important roles in earlier stage(s) of spermatogenesis. We went on to ask when the defect began and how the loss of Huwe1 led to such a defect. Spatial-temporal-histological analyses showed that the germ cell number was slightly decreased at dpp 3 (days postpartum), but dropped dramatically at dpp 6 . The proportion of gonocytes in the germ cell population is higher in the KO mice suggesting an arrest in the gonocyte stage. qPCR of SSC self-renewal and differentiating

marker genes confirmed that inactivation of Huwe1 affects both undifferentiating and differentiated spermatogonia lineage.

1.3.2.2 Direction of investigation and hypothesis

- *A potential role of Huwe1 in regulation of Forkhead box, class O (FoxO) 1*

Forkhead box, class O (FoxO) belongs to the large family of forkhead transcription factors which are downstream of insulin and insulin-like growth factor receptors. As a transcription factor, they control the expression of a variety of genes that regulate essential cellular processes, such as cell cycle, apoptosis, oxidative stress, atrophy, energy homeostasis, and glucose metabolism. Four FoxO members had been found in the mammalian genome: FoxO1, FoxO3a, FoxO4 and FoxO6[102]. They share a conserved forkhead box DNA-binding domain which possesses the ability to bind the FoxO-recognition element (FRE) sequence (G/C) (T/A)AA(C/T)AA. Phosphorylation is the major posttranslational regulator of FoxO function. All FoxO members except FoxO6 have regulatory AKT phosphorylation sites RxRxxS/T. This motif can be phosphorylated by AKT and other AGC family kinases such as PKA, PKC, SGK and PAK family kinases. Once phosphorylated, FoxOs will be exported from the nucleus into the cytoplasm. The phosphorylation sites serve as docking points for 14-3-3 binding which causes its retention in the cytoplasm and thereby abrogation of FoxO-mediated transcription. Subsequently, cytoplasmic FoxO appears to be rapidly turned over by ubiquitin-mediated degradation [102,103].

In general, FoxOs are thought to promote organismal longevity in invertebrates [104]. In line with this idea, FoxOs had been reported to regulate self-renewal in hematopoietic and neural stem cells [105,106]. More recently, Diego H. Castrillon demonstrated that FoxO1 is required in mouse

spermatogonial stem cells for their maintenance and the initiation of spermatogenesis [59]. In their study, FoxO1 knockout (refer as FoxO1 -/-) males were sterile. They have smaller testes, no spermatozoa were present in epididymides, despite the presence of round and elongating spermatids in most tubules. Histology analysis revealed that the number of spermatogonia were reduced by dpp 7, suggesting a defect in the proliferative expansion. Interestingly, they found FoxO1 expression undergoes cytoplasmic-to-nuclear translocation during development of gonocytes to spermatogonia. At P1, the protein was exclusively cytoplasmic, but nuclear translocation began at P3 and increased by P7. Moreover, they showed that the FoxO1 translocation is required for the induction of differentiation marker c-kit and regulates downstream target genes required for self-renewal including Dppa, Ret, Lhx1, Egr4, Sall4. Therefore, in the absence of FoxO1, SSC displayed a decreased ability to self-renew and the ability to initiate differentiation. Although FoxO3 and FoxO4 were previously shown to be dispensable for male fertility, in their study, the Triple FoxO knockout (refer as FoxO1/3/4 -/-) has a more severe phenotype than the single knockout. FoxO1/3/4 -/- males are infertile as well. Compared with those of FoxO1 -/- testes, FoxO1/3/4 -/- testes are much smaller and contain fewer germ cells and more tubules depleted of germ cells at all time points. It remains possible that FoxO3 and FoxO4 partially compensated for the FoxO1 deficiency.

Our Huwe1 -/- mice phenocopied the FoxO1/3/4 -/- mice. The adult males were totally infertile. The average weight of the testes of adult Huwe1 -/- mice was only 15% of the WT. Morphological analysis showed an intact somatic cell structure but complete abolishment of spermatogenesis. Spatial-temporal-histological analyses of germ cell markers Ddx4 and Tra98 showed a progressive loss of germ cell number in the KO mice. The germ cell number was slightly decreased at 3 dpp. However, at 6 dpp, when both the undifferentiated and the differentiating spermatogonia are

present, it dropped dramatically (~60%). Germ cell numbers fell in KO males to 12% of WT by 8 dpp when differentiating spermatogonia enter into meiosis and to 2% of WT by 15 dpp when spermatocytes are prominent in the first wave of spermatogenesis. As both undifferentiated and differentiating lineages are present at 6 dpp, we asked which lineage was affected by loss of Huwe1. qPCR and immunostaining of SSC self-renewal (Ngn3, Gfrα1, Plzf) and differentiated (c-Kit, Stra8 and Dazl) SSC markers revealed that inactivation of Huwe1 affects both lineages at dpp 6. Interestingly, quantification of FoxO1 stained germ cells revealed that the number of cells with cytoplasmic localization was higher in the Huwe1 KO by 36% compared to the WT. As FoxO1 is regarded as a transition marker of gonocyte to SSC, our results suggested a defect in SSC establishment from gonocytes. This result was confirmed by quantification of the percentage of germ cells that had migrated to the basement membrane which is another characteristic hallmark of gonocyte-to-spermatogonia transition. We found that only 46% of the germ cells in the KO had migrated to the basement membrane compared to 87% in the WT.

Interestingly, Western blot of testis lysate showed a decrease in the level of FOXO1 in our knockout mice. As FoxO1 is a pivotal regulator of SSC self-renewal and differentiation, I hypothesize that downregulation of FoxO1 upon Huwe1 inactivation causes a defect of SSC establishment from gonocytes which further affects spermatogenesis.

- *A potential role of Huwe1 in the DNA Damage Response*

DNA damage comes in many different forms including abasic sites, base modifications, single-strand and double-strand DNA breaks and DNA-protein cross links. Among these type of DNA damages, DNA double-strand breaks (DSBs) are considered to be the most cytotoxic. DSBs result from both endogenous sources such as replication fork collapse and exogenous sources such as

ionizing radiation (IR) [107,108]. Organisms have developed elaborate cellular pathways, the DNA damage response (DDR), to cope with DSBs [109,110]. DSBs can be sensed very quickly by various DSB 'sensor' proteins. The recruitment of sensor proteins activates different intracellular signaling pathways that culminate in cell cycle checkpoint arrest through inhibition of Cdks [111]. In general, if DSBs happen in S phase or G2 phase, the cell will conduct a G2/M arrest and utilize Homologous Recombination (HR), an error-free repair mechanism using the sister chromatids as template, to repair [112]. On the other hand, if it happens when there are no sister chromatids available such as in G1 phase, cells will utilize an error-prone mechanism Nonhomologous DNA End-Joining (NHEJ) for repair DSB. Normally, G1 arrest is associated with NHEJ [113].

For HR, the sensor MRN (Mre11-Rad50-Nbs1) protein complex binds to and unwinds the DSB ends [114,115]. NBS1 from MRN functions in recruiting and activating ATM which subsequently phosophorylates H2AX (on serine 139 also known as γ H2AX), a critical step in early cellular response to DSBs. MDC1 (mediator of DNA damage checkpoint protein 1) directly binds γ H2AX through its carboxyl-terminal BRCT repeats and potentiates the γ H2AX signal, by both promoting its phosphorylation and recruiting ubiquitin ligase RNF8. RNF8 possesses two critical domains: a FHA (forkhead-associated) domain which has binding specificity for phosphothreonine-containing epitopes and a RING domain which supports monoubiquitination. Through binding with phosphorylated MDC1 and phosphorylated HERC2 as well as mediating monoubiquitination of H2A and H2AX at DNA damage sites, RNF8 provides a critical link between phosphorylation and ubiquitylation events in the DDR [116]. RNF168 detects the monoubiquitin signal on histone H2A/H2AX and then carries out K63-linked ubiquitylation on residues K13/15, thereby promoting assembly of 53BP1 and BRCA1/RAP80 complexes at sites of DNA damage [117]. Besides being a repair mechanism, DDR initiates cell cycle arrest at G2/M in order to provide time for HR. This

involves two critical checkpoint kinases CHK1 and CHK2 which are required for activation and inhibition of a number of cell cycle regulators and tumor suppresser proteins such as p53 [107] [112].

For NHEJ, the sensing part is as for HR. Once the onsite H2AX is phosphorylated, it recruits Ku protein heterodimer (Ku70/Ku80) which binds tightly at each DSB termini. The Ku complex is ring-shaped and serves as a docking site for DNA PKcs (DNA-dependent protein kinase catalytic subunit). After DNA PKcs binds Ku proteins at both sides of DNA termini, they haul the termini close to each other. Two bridge-like domains from each DNA PK bind to each other and trigger its auto phosphorylation which catalyzes the connection of both DNA termini. This also prevents the DNA from a premature or erroneous DNA degradation. At this point, the Artemis nuclease is recruited by DNA PKcs (if needed for DSB resection). Artemis can be phosphorylated either by DNA PKcs or by ATM. Finally, the scaffolding protein XRCC4 allows the Ligase IV to bind to DNA and ligate both DSB ends fixing the DNA damage. Unlike HR which is mostly active in S phase and initiates G2/M arrest, NHEJ is active throughout the cell cycle, and its activity increases as cells progress from G1 to G2/M [107] [118] [113].

Compared with somatic cells, it is believed that SSCs possess a unique DNA damage response (DDR) machinery to protect the inheritable genome [119] [120]. Huwe1 has been reported to have a critical role in the mediation of genotoxic stress-induced apoptosis and DNA damage repair by regulating the stability of proteins such as CDC6 [91], BRCA1 [121], polymerase β (Pol-β) [122] or phosphorylation such as phosphor-ATM and phosphor-p53 [99]. However, its role in SSC has not been explored yet. Recently, it has been shown that the exit from quiescence provokes DNA-damage-induced attrition in hematopoietic stem cells [123]. We reasoned that similarly the gonocyte mitotic-re-entry would also elicit endogenous DNA damage, and loss of Huwe1 might cause a defect in repairing such DNA damage leading subsequently to cell death. First, my colleague

Rohini Bose carried out co-immunostaining of the DSB marker γ H2AX with germ cell marker Tra98 on postnatal testis sections to examine the DDR rate in germ cells. A significant increase in the number of intensively staining γ H2AX foci was observed in KO mice compared with WT littermates. However, the endogenous DNA damage rate was similar in the KO and WT cells as revealed by comet assay performed by my colleague Dr. Kin Lam Fok. From cell cycle analysis, we noted a cell cycle arrest at G2/M phase of $CD9^+$ spermatogonia from KO mice. G2/M arrest is another hallmark of DDR which concomitantly induces HR. Collectively, these data showed that loss of Huwe1 did not affect endogenous DNA damage rates but caused an abnormal DDR.

I hypothesize that, upon Huwe1 inactivation, the excessively phosphorylated H2AX might be caused by a hyper-activated sensor or serine kinase. Alternatively, it might be caused by the failure to remove the upstream DNA damage signal (γ H2AX) because loss of Huwe1 might interrupt downstream steps of DNA repair.

1.3.3 Summary of objectives and results.

The overall objective of my work was to study the potential role of Huwe1 in the regulation of SSC and to find its direct targets in the SSC. Specifically, 1) As Western Blot revealed a decreased expression of FoxO1 in neonatal KO testis, my objective was to explore how Huwe1 regulates FoxO1, whether through regulating its stability or indirectly regulating its transcription. Moreover, I wanted to know whether the downregulation of FoxO1 is responsible for the phenotype. 2) As we observed increased γ H2AX foci in germ cells, I wanted to understand the underlying mechanism, whether loss of Huwe1 led to a hyperactivation of DDR or a failure of γ H2AX removal caused by a defect in the repair mechanism.

CHAPTER 2 MATERIALS AND METHODS

2.1 Animals

Conditional Huwe1 knockout (Huwe1$^{flox/flox}$) mice were generated as previously described. To initiate inactivation of Huwe1 specifically at embryonic day (e)15-e18 or postnatal day (dpp)3, Huwe1$^{flox/flox}$ females were bred with male mice hemizygous for Ddx4-Cre (FVB-Tg(Ddx4-Cre)1Dcas/J and STOCK Tg(Stra8-icre)1Reb/J (The Jackson Laboratory) respectively. Huwe1$^{flox/Y}$ (WT) and Huwe1$^{-/Y}$ Ddx4-Cre (KO) male offspring were identified by genomic PCR on tail DNA using oligonucleotides derived from the Cre recombinase sequence (Table 2 Oligonucleotides used in these studies). To measure cell proliferation in vivo, mice were injected intraperitoneally with 50 μg/g BrdU in saline at 2 dpp and sacrificed 24 hr later. All procedures were carried out in accordance with the regulations of the Canadian Council for Animal Care and were approved by the Animal Care Committee of McGill University.

2.2 Cell cultures

The C18-4 spermatogonial type A cell line was cultured at 34˚C with 5% CO2 in DMEM supplemented with 5% FBS, 2 mM L-glutamine, 1x non-essential amino acids (Invitrogen), and 1 mM sodium pyruvate [124].

1) siRNA knockdown experiment. C18-4 cells (1.5 X 10^5 cells) were plated in each well of a 6-well plate one day before transfection. The next day, 100 nM Huwe1 siRNA or nonspecific control siRNA oligos (ordered from IDT) were transfected using Lipofectamine RNAiMAX following the manufacturer's protocol. After 48 hours and 72h of transfection, cells were harvested by trypsinization for cell count as well as RNA/protein extraction.

2) Overexpress FoxO1 upon Huwe1 siRNA silencing. siRNA silencing was performed as described above. pCMV5-HA FoxO1 plasmid (Addgene #14936) was transfected the day after siRNA transfection. Cell number was determined 48 hours after FoxO1 transfection (i.e. 72 h transfection after siRNA transfection). Cells were harvested by trypsinization for RNA and protein extraction.

3) CRISPR/Cas9 nuclease mediated Huwe1 Knockout experiment. Single guide RNAs targeting either exon 14 or 15 (Table 2 Oligonucleotides used in these studie) were cloned into LeGO-U6-Cas9 vector using AgeI and SphI (New England Biolab) or into pSpCas9(BB)-2A-Puro (Addgene ID 48139) using BbsI (New England Biolab). Lentivirus were generated by cotransfecting LeGO-U6-Cas9 vector with psPAX2 (Addgene ID 12260) and pCMV-VSV-G (Addgene ID 8454) into 293T cells with Lipofectamine 2000 (Invitrogen). Cells ($8x10^6$) were seeded together with DNA:liposome complex on a 0.0001% poly-L-lysine pre-coated plate in DMEM medium supplemented with 10% FBS. Twenty four hours later, the medium was changed to virus harvesting medium (DMEM, 10% FBS, 2 mM L-glutamine). Virus supernatant was collected 48 hrs post-transfection and concentrated 10-fold by ultracentrifugation at 110,000 x g for 90 mins.

C18-4 cells ($1x10^6$) were seeded on a 60 mm dish the night before transduction. The cells were transduced at 30-40% confluence with 200 µl LeGO lentivirus in the presence of 8 µg/ml polybrene [124]. Virus-containing medium was changed to normal culture medium 16-20 hours after transduction. Transduced cells were isolated by fluorescent-activated cell sorting (FACS) for the GFP positive population. The T7 endonuclease assay {Lin:2014tr} was used to confirm specific gene inactivation. On target sites and off target sites in genomic DNA were amplified by PCR. Purified PCR products (300 ng) were denatured at 95˚C for 10 min and subjected to step-down annealing from 85 to 25˚C. Annealed products were digested with 0.5 U of T7 endonuclease I

(New England Biolab) for 1 hr at 37˚C. Reactions were quenched with EDTA (final concentration 45 mM) and separated by agarose gel electrophoresis.

4) FoxO1 overexpression upon CRISPR/Cas9 nuclease mediated Huwe1 Knockout. We subcloned FoxO1 into lentivirus pLVX-IRES-mCherry vector (Clontech). Both virus containing FoxO1 and CRISPR/Cas9 were harvested as described above. GFP (CRISPR vector) and mCherry (FoxO1 vector) double positive cells were were sorted by FACS and reseeded into 24 well plate at $1x10^5$ per well. Cell number was determined 5 days after seeding. Cells were harvested by trypsinization for protein extraction.

2.3 Long term SSC Culture

Primary SSC culture was established as described from Huwe1flox/Y testes. Briefly, a single cell suspension of testicular cells was prepared using a two-step enzymatic procedure with collagenase and trypsin. These cells were enriched for SSC by magnetic-activated cell sorting using anti-Thy1 antibodies and seeded onto STO feeder layers in serum free medium [MEMα medium (Invitrogen), 0.2% BSA, 5 μg/ml insulin, 10 μg/ml iron-saturated transferrin, 7.6 μq/L free fatty acids, 3x10-8 M Na2SeO3, 50 μM β-mercaptoethanol, 10 mM HEPES, 60 μM putrescine (all from Sigma), 2 mM L-glutamine, 1x penicillin-streptomycin (both from Invitrogen)] supplemented with 20 ng/ml recombinant human GDNF, 100 ng/ml recombinant rat GFRα1 Fc chimera (both from R&D system) and 1 ng/ml basic FGF (BD Biosciences). STO cells were seeded onto collagen (0.1%) coated 24-well plates at 1 x 10^5 cells 1-3 day before and grown at 37 °C with 5% CO2 in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin- streptomycin). SSC were incubated at 37˚C with 5% CO2 and subcultured every 5-6 days.

To perform tamoxifen inducible inactivation of Huwe1 in the SSC culture, CreERT2 was amplified by PCR from pMSCV CreERT2 puro (Addgene ID 22776) and subcloned into the EcoRI and XbaI sites in the pLVX-EF1α-IRES-mCherry plasmid (Clontech). Lentivirus was generated with this plasmid as described above for the LeGO plasmid except that the virus supernatant was concentrated by 20-fold. For transduction, Huwe1flox/Y SSC were seeded at a density of $5x10^4$ cells/cm^2 in each well of a 24-well plate. Two days after seeding, 100 µl lentivirus was added to the culture together with 6 µg/ml polybrene [126]. The culture was centrifuged at 2000 rpm for 90 min to facilitate transduction. Virus-containing medium was changed to normal culture medium 16-20 hrs after transduction. Transduced cells were sorted by FACS for mCherry positive cells. To inactivate Huwe1, these stably transduced SSC were seeded at a density of 7.5x104 cells/cm2. Two days later, cells were exposed to 1 µM 4-hydroxyl tamoxifen [127]. The medium was changed the next day and cells were collected at the indicated time points.

2.4 Immunoblot Analysis

Aliquots of cell preparations were solubilized in RIPA buffer (150 mM NaCl; 50 mM Tris-Cl pH 8; 1% NP-40; 0.5% deoxycholate; 0.1% SDS) with protease inhibitor cocktails (Roche). Protein concentration was quantified using Bio-Rad protein assay reagent following the manufacturers' protocol. Proteins (30 ug) were separated on 7% or 15% Tris-glycine gels. The gels were then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad; Hercules, CA). After blocking with 5% milk in 1xTBST the membranes were incubated with specific primary antibodies (Table 1 Antibodies used in these studies) diluted in TBST overnight at 4°C, followed by incubation with a horseradish peroxidise-coupled secondary antibody for one hour and then ECL-

enhanced chemiluminescence (GE Healthcare, USA). Blots were quantified using Quantity One software (Bio-Rad). Tubulin was used as loading reference.

2.5 RNA extraction and RT-PCR analysis

Total RNA was extracted from cell pellets using Trizol（Life technology） reagent and digested with DNase I (Qiagen, Santa Clarita, CA). cDNA was synthesized from the isolated RNA using the High Capacity cDNA Kit (Life Technologies). Expression of various ubiquitin-related genes, SSC marker genes were examined by real time (RT)-PCR. The reactions were carried out using SYBR® Green Master Mix and amplified using ViiA™ 7 system. PCR cycle conditions were 95°C for 10 min; 40 cycles of 95°C for 15 sec, 52°-58°C (depending on primer set) for 30 sec, and 72°C for 30 sec. This was followed by melting curves and cooling cycles. The comparative CT method was used to analyze the data. Simultaneous runs of the samples were performed using GAPDH as a housekeeping gene. Assays were performed in triplicate. For each treatment condition or cell type studied, the mRNA levels were determined in samples from three to four independent cell preparations. The results were expressed as the means ± SEM of the fold changes in relative expression levels normalized to GAPDH.

2.6 Immunostaining

Protein expression of DNA damage sensor and DDR regulators was examined by immunocytochemistry on paraffin embedded sections of postnatal testis. Briefly, sections were deparaffinized (3x5 min 100% Citrosolv), then rehydrated (2x5 min 100% Ethanol, 1x3 min 95% Ethanol, 1x3 min 70% Ethanol,1x5min water) followed by antigen retrieval (Slides were placed in citrate buffer (10mM Citric acid, 0.05% Tween 20, pH 6.0) and boiled in the microwave for 6

min at power 100. Following cooling of slides for 2 min, the slides were reheated for 10 min at power 60. Slides were then cooled for 20 min.) The sections were blocked for 1-2 h using blocking solution in a humidified chamber at room temperature. (blocking buffer: 800 ul 1XPBS+100 ul 10% BSA+100 ul goat serum+sodium azide 0.02%). The slides were incubated overnight at 4°C with varying primary antibodies (Table 1 Antibodies used in these studies). The next day, the slides were washed with 1XTBST and then incubated with secondary antibody dissolved in PBS-Tween (1x PBS+ 0.5% Tween 20) at a concentration of 1:100 for 2 hours in a humidified chamber at RT. The slides were washed with TBST and counterstained with Hoechst 33342 (Sigma) for 30 min at room temperature. Slides were mounted with anti-fade solution. Slides were stored in the dark at 4 C^o before examination by florescence microscopy or at -20 C for long term storage. Negative controls were done by incubating some samples with nonimmune rabbit IgG (Invitrogen). Representative results are shown.

2.7 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.0 (GraphPad Software). Student t test was used for analysis involving two groups of samples. One way ANOVA was used for analysis involving ≥ 3 groups of samples. Two way ANOVA was used for analysis involving more than one independent variable. $P<0.05$ was considered as significant.

CHAPTER 3 RESULTS

3.1 An investigation into the role of Huwe1 in FoxO1 stability

Depletion of Huwe1 either inhibits cell proliferation or leads to cell degeneration

In order to study the role of Huwe1 in spermatogenesis, I depleted Huwe1 in the Type A spermatogonia cell line C18-4 by using siRNA silencing (KD) or CRISPR/Cas9 system (KO)（Figure 7 A). For the KD experiment, we tested 4 pairs of oligos (Table 2 Oligonucleotides used in these studies) and chose si214 as it had the best knockdown efficiency. For the KO experiment, two pairs of sgRNAs targeting exon 14 or 15 of Huwe1 (referred to as g14B and g15B) were designed and major off-target sites were ruled out by T7 endonuclease assay. Western blot analysis confirmed successful silencing (KD) of Huwe1 72 h after siRNA transfection (Figure 7 B) and successful inactivation (KO) of Huwe1 by both sgRNAs (14b and 15b) 4 days after reseeding (Figure 7 C). In keeping with reports by others [91] [124], when we cultured cells lacking Huwe1 for three days, we noted a significant proliferation defect shown as a reduction in cell number at the time point I harvested the cells (Figure 7 D - F). Therefore, we believe that depletion of Huwe1 in C18-4 cells is a good model that mimics our in vivo observations in Huwe1 KO mice.

Loss of Huwe1 destabilizes FoxO1 protein

To determine whether loss of Huwe1 in the cell line would result in decreased FoxO1 as seen in the 3dpp KO mice testes (Figure 8 A), we measured FoxO1 levels in the KD and KO cells. Western blotting showed a 30-40% decrease in FoxO1 upon loss of Huwe1 indicating the validity of our cell model. (Figure 8 B, D). Huwe1 may modulate either the synthesis or the degradation of FoxO1.

qRT-PCR revealed that FoxO1 mRNA levels were similar in KD and KO cells compared to control cells suggesting that Huwe1 does not affect FoxO1 at the transcriptional level (Figure 8 C). To measure rates of degradation of FoxO1, I treated control cells or Huwe1-depleted cells with cycloheximide (CHX) to block new protein synthesis and monitored the FoxO1 level at different time points. The rate of disappearance of FoxO1 was increased upon Huwe1 silencing (Figure 8 E). Collectively, these results suggested a protective role of Huwe1 toward protein stability of FoxO1.

Restoration of FoxO1 is insufficient to reverse the cell depletion upon loss of Huwe1

To evaluate whether the loss of FoxO1 is responsible for the decrease in cell number upon Huwe1 depletion, I tested whether overexpression of FoxO1 would reverse the defect.(Figure 9 A). Transient transfection of KD C18-4 cells with plasmid expressing FoxO1 or of KO C18-4 cells with lentivirus expressing FoxO1 resulted in a 10 fold overall increase in FoxO1 levels in the cells (Figure 9 E and data not shown), but unexpectedly led to a further impairment of cell growth. (Figure 9 B, C). Since FoxO1 can be a pro-apoptotic factor[125], the marked overexpression may have increased cell death. However, in another experiment performed by the post doctoral fellow Dr. Fok in which SSC depleted of Huwe1 upon tamoxifen activation of Cre-recombinase were transduced with lentivirus expressing FoxO1, the resulting FoxO1 levels in the KO SSCs were restored only to normal levels. Nonetheless, cell growth was also further impaired in these cells. Besides, to assess whether the transcriptional activity of FoxO1 was impaired as a result of the decreased FoxO1 levels in the Huwe1 depleted C18-4 cells, I measured mRNA levels of several FoxO1 target genes by qRT-PCR. None of those genes showed decreased expression in cells lacking Huwe1, while we surprisingly observed that p21 and CCND2 were elevated in the KD and

KO system respectively. Together, these studies would suggest (Figure 9 F). that FoxO1 might not be a mediator of the effects of Huwe1 on spermatogonial development.

3.2 An investigation into the role of Huwe1 in the DNA damage response

Loss of Huwe1 in SSC does not affect DDR regulators as well as some known substrates of Huwe1

To test whether loss of Huwe1 affects the DDR in SSCs, we conditionally inactivated Huwe1 in primary SSC culture using tamoxifen-inducible CreERT2. First, we asked whether Huwe1 depletion causes the sensor hyper-activation. Total cellular protein of inducible Huwe1 KO and WT SSC were subjected to Western blot analysis. Immunoblotting for phospho-ATM or NBS1 (from MRN complex) revealed that these two DDR upstream factors seemed not to be affected in the KO SSC cells (Figure 10) suggesting the sensing part of the DDR is intact. Next, we asked whether other DDR regulators were affected by loss of Huwe1. I probed other regulators throughout the DDR pathway including ATR, RNF8, RNF168, RAD51, CDC25A, phosphor-CHK2 and CDC6. None of them showed consistent changes upon Huwe1 KO (Figure 10). Notably, phospho-ATM, RAD51, phospho-CHK2 levels were quite low indicating a comparatively low endogenous DDR response (data not shown). The levels of some well known substrates of Huwe1 including p53 and MYC were also similar in WT and KO SSC. All together, loss of Huwe1 in SSC doesn't affect well-known DDR regulators and it suggests a unique function and different substrates of Huwe1 in SSC compared with somatic cells.

Loss of Huwe1 causes defects in DDR of SSC upon inducing DNA damage

Another possibility is that the increased γ-H2AX foci that we observed in vivo is a consequence of the failure of DNA damage repair upon Huwe1 depletion. As SSCs grow very slowly, it was hard to get enough cells to expose to different treatments. We therefore asked whether the C18-4 KD model is a suitable in vitro model. Immunostaining of γ-H2AX revealed a similar increased basal level γ-H2AX foci in KD cells compared with control cells (Figure 11 A). As C18-4 KD cells mimic the in vivo observation, we regarded it as a suitable model to study the DDR in vitro. It is widely accepted in DDR research that the number of γ-H2AX foci as well as the level of H2AX phosphorylation observed by IF or WB are informative about the extent of DNA repair. Therefore, we asked whether the removal rate of γ-H2AX is affected in Huwe1 depleted cell. We challenged C18-4 cells with hydroxyurea (HU), a drug that induces DSBs by depleting the cells of dNTPs, resulting in stalled replication forks collapse and performed a time course of Western blot analyses of γ-H2AX, phospho-CHK2 and phospho-CHK1 (Figure 11 B). Cells were harvested at different time points during HU treatment and recovery time and total cellular proteins were subjected to Western blot analysis. In line with our previous conclusion that the DDR sensing is intact, the induction of γ-H2AX by HU seems identical in both control and KD from time 0 to 1 h. However, KD cells retain high γ-H2AX level up to 8 h after HU was removed whilst γ-H2AX was halved within 3 h in control siRNA cells. Notably, the ubi-H2AX appeared faster in KD than control cells suggesting that Huwe1 might also regulate the ubiquitin signaling cascade of DDR. We also note a blunted induction of phospho-CHK2 in KD cell and the pattern of CHK1 induction seemed altered as well. Taken together, these results confirmed that loss of Huwe1 causes a defect in the DDR.

CHAPTER 4 DISCUSSION

In this thesis, I conducted studies to explore possible mechanisms to explain the critical requirement for Huwe1 in spermatogonial development. The decreased levels of FoxO1 seen upon inactivating Huwe 1 in both mouse testis and cultured spermatogonia cell line suggested that it might be involved. This decision to study FoxO1 was based on a previous report from the Castrillon's laboratory demonstrating an important role for FoxOs in spermatogonial development. However, KO of FoxO1 had only a partial deficiency in spermatogenesis and a KO of all three FoxO isoforms – 1, 3, 4 – was required to produce a phenotype similar to that generated by our Huwe1 KO. In our case, the decrease in FoxO1 caused by Huwe1 depletion was only ~30%. Whether other FoxOs were also decreased by loss of Huwe1 remains to be studied. This modest depletion of FoxO1 that we observed as well as the failure to reverse the phenotype in our C18-4 cell model by overexpressing FoxO1 suggests that the decrease in FoxO1 may only be partially responsible for the impaired cell growth. Indeed, as described in the introduction, Huwe1 has many substrates that are involved in cell proliferation and cell death (e.g. p53, Myc, Mcl-1, Cdc6) and so it is quite possible that correcting several targets in addition to FoxO1 deficiency may be required to reverse the phenotype that we observed. However, to date, I have tested whether expression of c-MYC, p53 and CDC6 are altered upon loss of Huwe1 in SSC, but have not seen any differences in these substrates.

Although the importance of FoxO1 in mediating the effects of Huwe1 remains unclear, we consistently saw a decrease in its level in different Huwe1 KD/KO models in vivo and in cell culture which appears to be due to enhanced degradation of FoxO1. Therefore, there is definitely a protective function of Huwe1 towards FoxO1, but the underlying mechanism remains unclear. It is possible that Huwe1 prevents phosphorylation and subsequent degradation of FoxO1 by either

directly binding to it or by ubiquitinating it in such a manner that it prevents the ability of Akt kinase to act on it. This could be explored further by testing whether FoxO1 co-immunoprecipitates with Huwe1 and whether levels of pFoxO1 are increased in KD/KO cells. If such an interaction of FOXO1 with HUWE1 is confirmed, the domain(s) responsible for the interaction could be identified and assessed as to whether they are sites for or can modulate phosphorylation or ubiquitination.

Another possibility is that Huwe1 down-regulates an E3 ubiquitin ligase that is responsible for FOXO1 degradation. So far, three ubiquitin ligases for FOXO1 have been identified [125-127]. They are SKP2, COP1 and MDM2. Among them, MDM2 is the most likely candidate regulator of FoxO1 in our KO system. MDM2 is a well-known E3 ubiquitin ligase for p53, promoting its ubiquitination and degradation. Mdm2 itself is transcriptionally upregulated by p53 thereby forming an autoregulatory feedback loop. A paper from Bai's laboratory indicates that upon DNA damage, the interaction between MDM2 and p53 is suppressed, resulting in increased p53 and FOXO1 activities triggering apoptosis. Another report showed that HUWE1 can be polyubiquitinated and degraded by MDM2 suggesting that HUWE1 and MDM2 cannot be highly expressed at the same time. Thus, it is possible that in the absence of HUWE1, up-regulated p53 may induce MDM2 expression that leads to a decrease in FoxO1 protein. To test this hypothesis, we would need to probe these E3 ligases by Western blotting in the C18-4 model or SSC model. Notably, SKP2 and COP1 does not only regulate FoxO1 but also other FoxOs and so these ligases would be implicated if I had also observed down-regulation of other FoxOs upon loss of Huwe1.

On the other hand, the study of the role of Huwe1 in the DDR was based on the observation of increased γ-H2AX staining in neonatal KO testis. H2AX is phosphorylated and concomitantly γ-H2AX foci are formed within seconds after induction of DSBs. γ-H2AX foci become stable and

can be visualized by immunofluorescence 15–30 min later [128]. Once the repair of DSBs commences IRIF (Irradiation induced foci) with the recruitment of other proteins such as MDC-1, RNF8, P53BP1 within 3 h, γ-H2AX will be gradually removed by dephosphorylation or degradation. We hypothesized that loss of Huwe1 causes either a hyperactivation of DDR sensing or a failure to remove γ-H2AX. Unchanged levels of phospho-ATM and NBS1 in SSCs rules out the first possibility. Then we recapitulated the increased γ-H2AX staining on Huwe1 depleted C18-4 cell at the basal level. Challenging C18-4 cells with HU to induce DSBs showed similar induction of γ-H2AX in both control and Huwe1 KD cells. However, the KD cells removed γ-H2AX less efficiently after the media was switched back to normal. This result supports our second hypothesis that the loss of Huwe1 caused a failure of γ-H2AX removal.

The underlying mechanism of how Huwe1 depletion causes failure of γ-H2AX removal remains unclear. As γ-H2AX removal rate is an indicator of successful repair of DSBs when cells encounter DNA damage, it is possible that loss of Huwe1 blocks the DDR pathway at certain step(s). As the downstream mechanism failed to complete DSBs repair, the upstream pathway retained γ-H2AX at DSB sites in order to keep pushing DDR signaling. However, unexpectedly, Western blotting of several DDR regulators including some Huwe1 potential targets showed no change between WT and KO SSCs, suggesting that loss of Huwe1 might indirectly block the DDR instead of directly regulating the stability of DDR proteins. CHK2 is a checkpoint kinase that is phosphorylated and activated in response to DNA damage induced by ionizing radiation (IR), UV irradiation, and replication block by hydroxyurea[129]. Interestingly, we noted that a blunting of induction of phospho-CHK2 upon HU treatment and IR-irradiation (data not shown) on C18-4 KD cell and UV treated SSC Huwe1 KO cell (data not shown). Blunted Chk2 phosphorylation leads to an insufficient phosphorylation on Cdc25C on Ser216 which further interferes with the

activation of Cdc2 and might cause a G2/M arrest [130,131]. In line with this idea, my colleague observed a G2/M arrest in Huwe1 KO SSCs (data not shown). We reasoned that such cell cycle arrest caused the mitotic catastrophe which gradually depleted germ cells in vivo. Long term live imaging of cultured C18-4 cells revealed delayed cell division and unexpected degeneration of Huwe1 KO cells (Cell Reports paper in revision). It is known that phosphorylation of Chk2 is condition-dependent: In response to IR CHK2 phosphorylation is ATM-dependent; In response to UV or HU, Chk2 phosphorylation is ATM-independent. Further study is needed to understand how Huwe1 regulates phospho-Chk2.

The second possibility of how loss of Huwe1 causes the γ-H2AX retention is that Huwe1 directly affected the γ-H2AX stability. We previously identified all four core histones as in vitro substrates of Huwe1. It is quite possible that Huwe1 can also guide ubiquitination and degradation of the H2Ax variant. Therefore, the absence of Huwe1 might cause a defect in H2AX degradation. A paper published recently by Hirobumi Teraoka's group supports this hypothesis. Their study concluded that in normal somatic cells H2AX is continuously produced but does not accumulate due to degradation via poly-ubiquitination by HUWE1. However, upon DNA damage, phosphorylation of H2AX on Ser 139 (γ-H2AX) prevents its ubiquitination by Huwe1 and therefore enables efficient γ-H2AX foci formation and DSB repair. One straight forward way to test this hypothesis is to block the UPS with the proteasome inhibitor MG132 in both control and KD C18-4 cells, and to see whether the γ-H2AX degradation is blocked in control cells. We would also need to test the interaction and ubiquitination activity of Huwe1 and H2AX in C18-4 cells as the germ cells may behave differently from somatic cells.

Finally, it is also possible that Huwe1 positively regulates the stability of a phosphatase which is responsible for γ-H2AX removal at the DSB site. A previous study found that protein phosphatase

2A (PP2A) is involved in the removal of γ-H2AX foci independently of ATM, ATR, or DNA-PK activity. Moreover, such removal is necessary and facilities the DNA repair. It is possible that loss of Huwe1 destabilizes PP2A, causing the retention of γ-H2AX at DSBs sites which further inhibits DNA repair and causes cells to be hypersensitive to DNA damage. To test this hypothesis, we would need to probe levels of PP2A by Western blotting in the C18-4 model or SSC model.

As a major protein turnover mechanism in eukaryotes, UPS involves numerous biological processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis. Our observations on the functions of Huwe1 contribute to the growing body of evidence implicating the UPS in important functions of spermatogenesis. Notably, our results implicate this system early in this process, as early as gonocytes. Its cellular functions include regulating key steps in the DNA damage repair response. In the germ cells, the defective response appears to lead to mitotic catastrophe rather than apoptosis (Cell Reports paper in revision).

Infertility as well as subfertility affect a significant proportion of humanity and have become a major public health issue. The molecular pathology remains largely unknown. Interestingly, our collaborator Dr. Wenming Xu in China has identified a SNP in the Huwe1 promoter that appears associated with azoospermia. If confirmed, it would indicate that some cases of male infertility may be due to defective Huwe1 expression and this SNP could then be a useful biomarker.

FIGURES

Figure 1

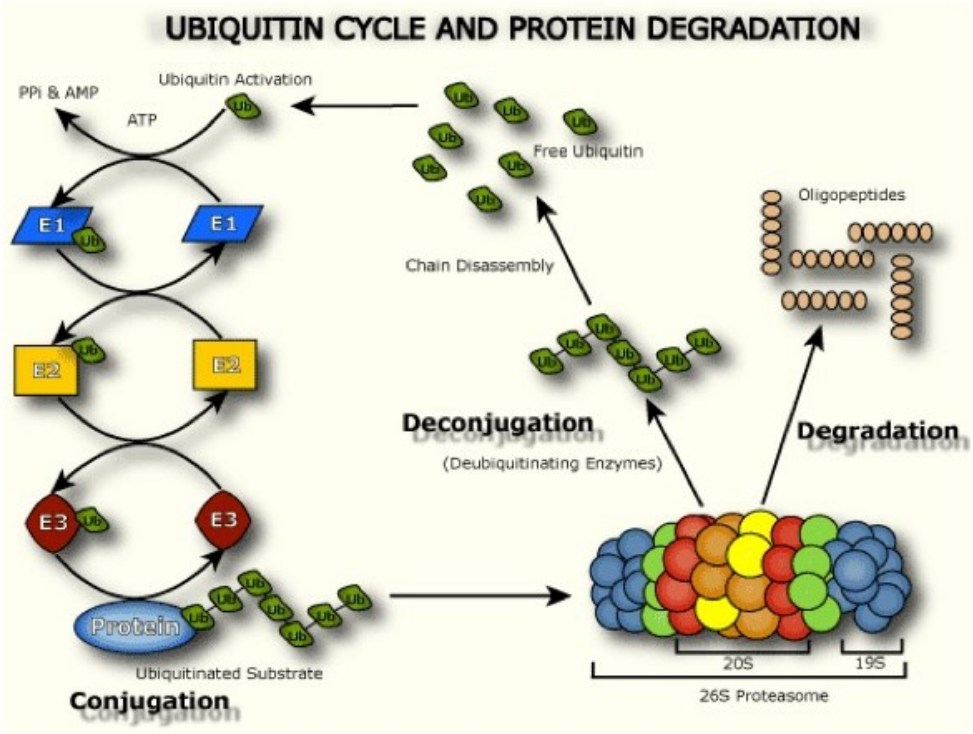


Figure 1 Overview of signaling by ubiquitin.
(*http://web.archive.org/web/20080330210016)*

Figure 2

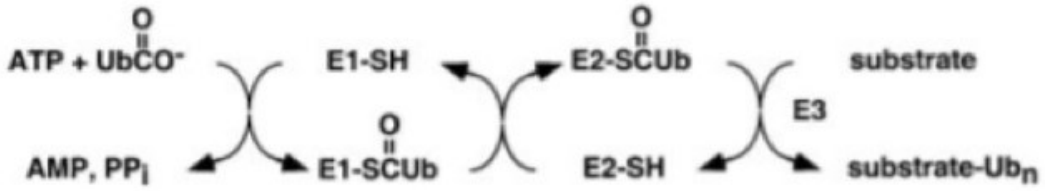


Figure 2 Enzymatic pathway of ubiquitin conjugation (Cecile M. Pickart Mechanisms underlying ubiquitination 2015 [13])

Figure 3

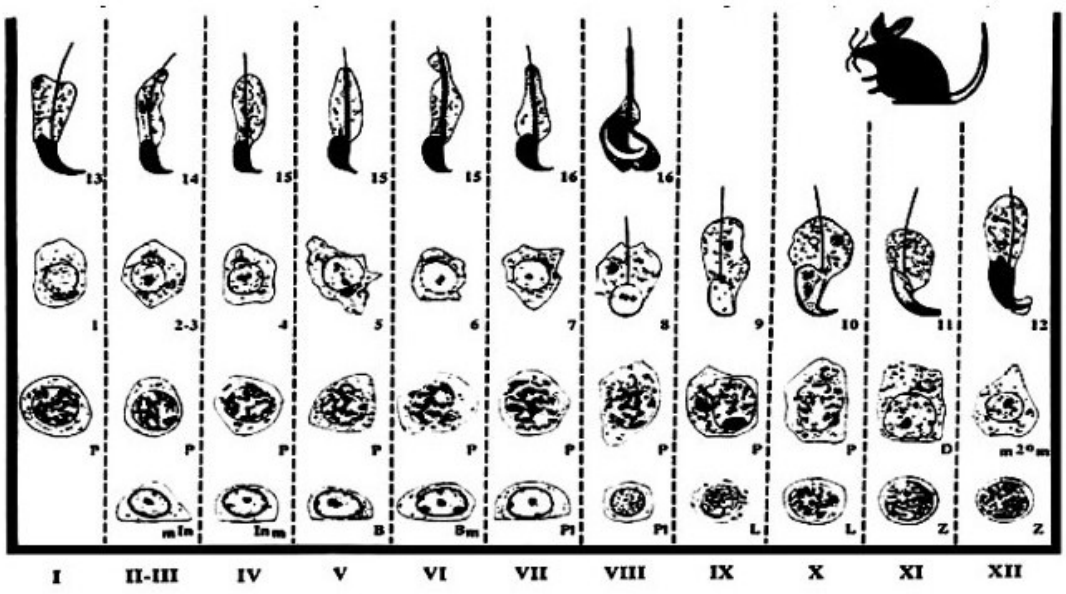


Figure 3 Diagram of the 12 stages for the production of spermatozoa in the mouse seminiferous epithelium (Russell et al. 1990 [13])

Figure 4

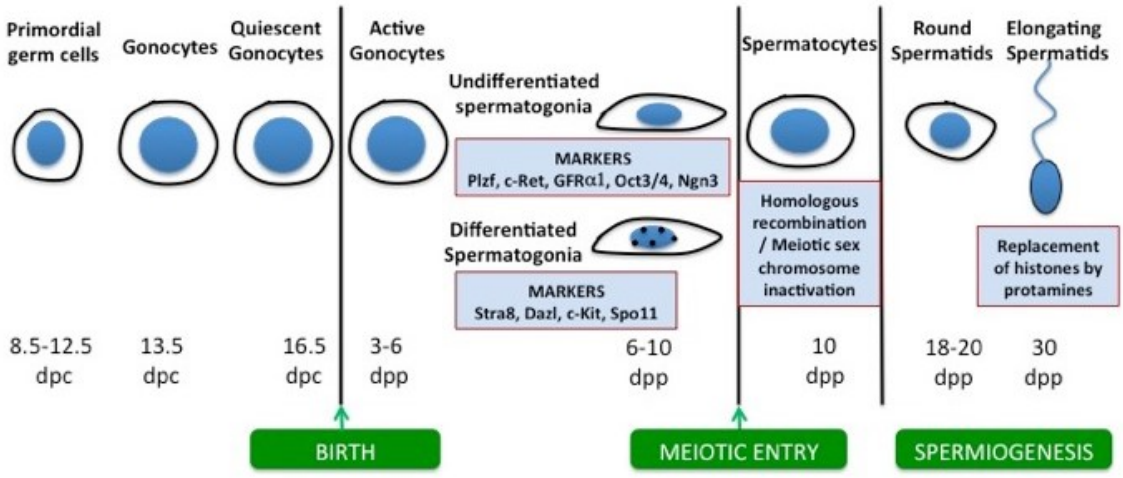


Figure 4 Overview of spermatogenesis (Rohini Bose, Phd Student from Simon Wing's Lab)

Figure 5

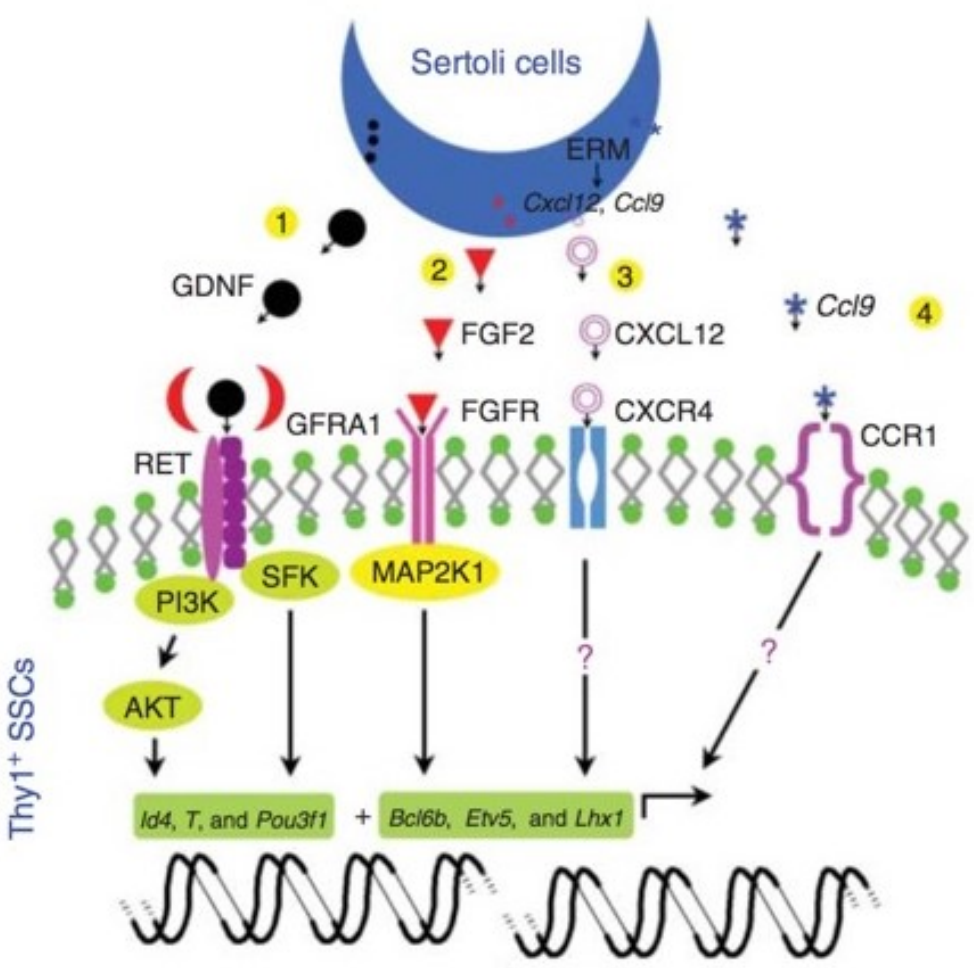


Figure 5 Current understanding of signaling pathways regulating SSC self-renewal in mouse testis.

Figure 6

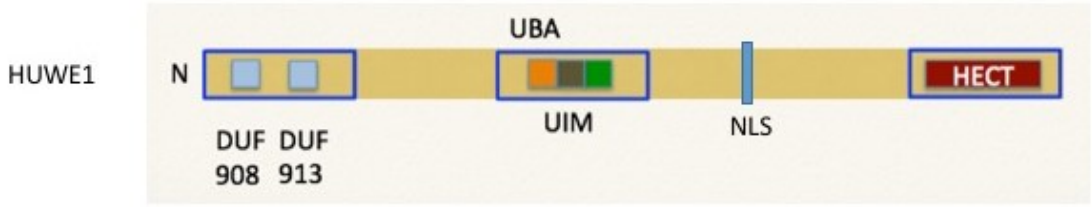
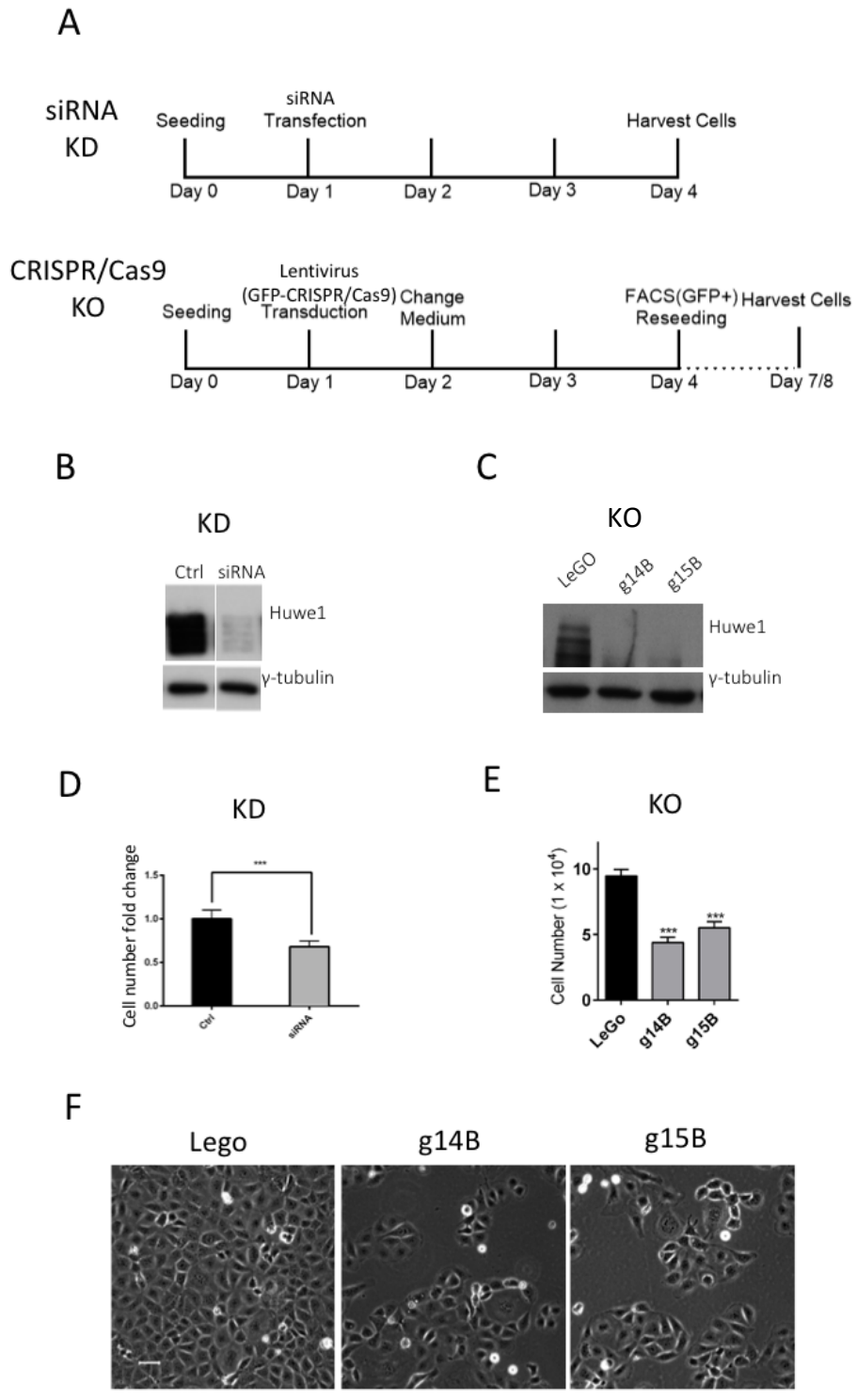


Figure 6 Schematic representation of Huwe1 gene and protein domains.

Figure 7


A
siRNA KD
Seeding
siRNA Transfection
Harvest Cells
Day 0
Day 1
Day 2
Day 3
Day 4
CRISPR/Cas9 KO
Seeding
Lentivirus (GFP-CRISPR/Cas9) Transduction
Change Medium
FACS(GFP+) Reseeding
Harvest Cells
Day 0
Day 1
Day 2
Day 3
Day 4
Day 7/8
B
KD
Ctrl
siRNA
Huwe1
γ-tubulin
C
KO
LeGO
g14B
g15B
Huwe1
γ-tubulin
D
KD
Cell number fold change
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Ctrl
siRNA
E
KO
Cell Number (1 x 10⁴)
***
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LeGo
g14B
g15B
F
Lego
g14B
g15B

Figure 7 Depletion of Huwe1 results in impaired cell growth.

A. Outline of the establishment of Huwe1 KD and KO C18-4 cell line by siRNA and CRISPR/Cas9 technology. B,C. Effectiveness of Huwe1 depletion confirmed by Western blotting. D,E Loss of Huwe1 in C18-4 cells results in decreased cell number. Each experiment employed triplicate wells. N=4 (KD), N=2 (KO). F Phase contrast images of the indicated groups 4 days after seeding (left panel; scale bar = 50 μm).

Figure 8

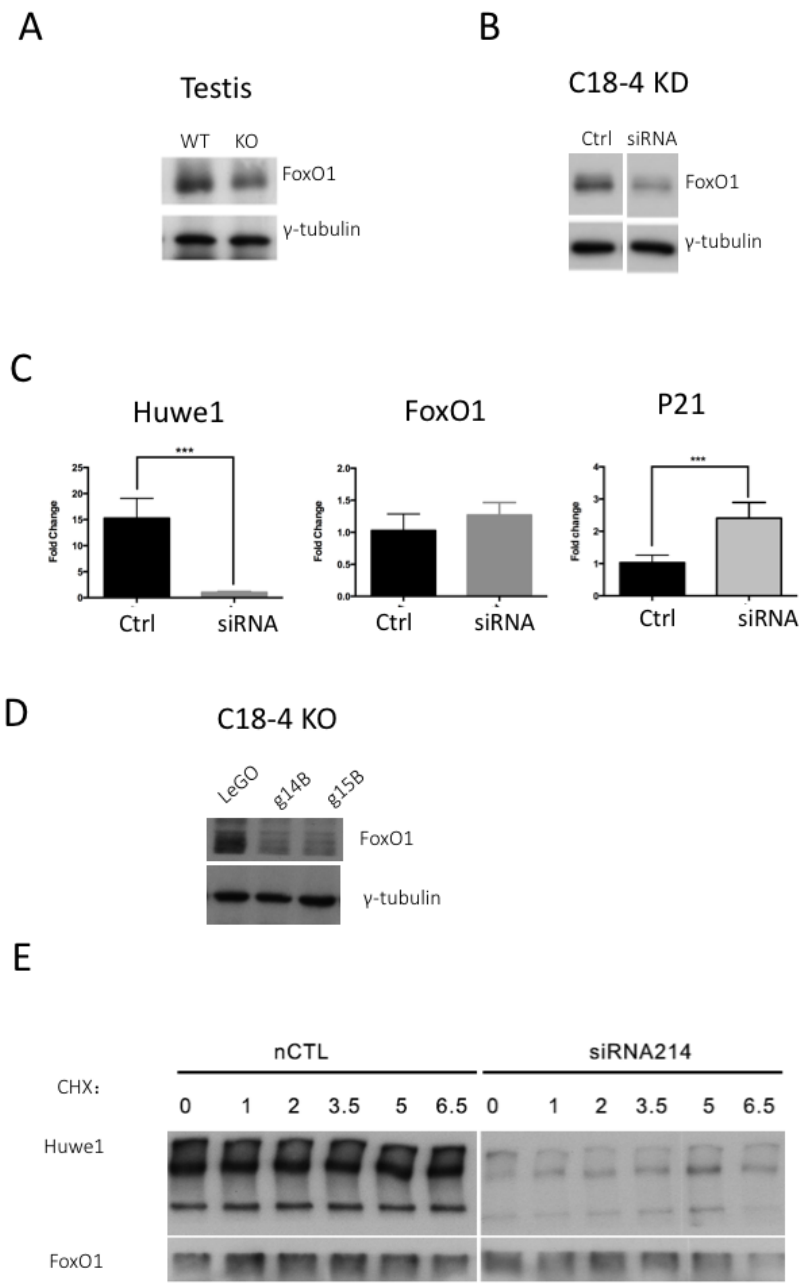

Figure 8 Loss of Huwe1 destabilizes FoxO1 at protein level.

A. Western blot of FoxO1 from WT and KO adult testis protein.(n=2). B,D. Downregulation of FoxO1 upon Huwe1 KD and KO (g14B, g15B guide RNAs) as shown by Western Blotting. (n=5). C. Quantitative RT-PCR analysis of Huwe1, FoxO1, p21 from C18-4 cell KD and control cells. E. Rates of degradation of FoxO1. Western blot of FoxO1 following treatment of cells with CHX for the indicated time points (hrs).

Figure 9

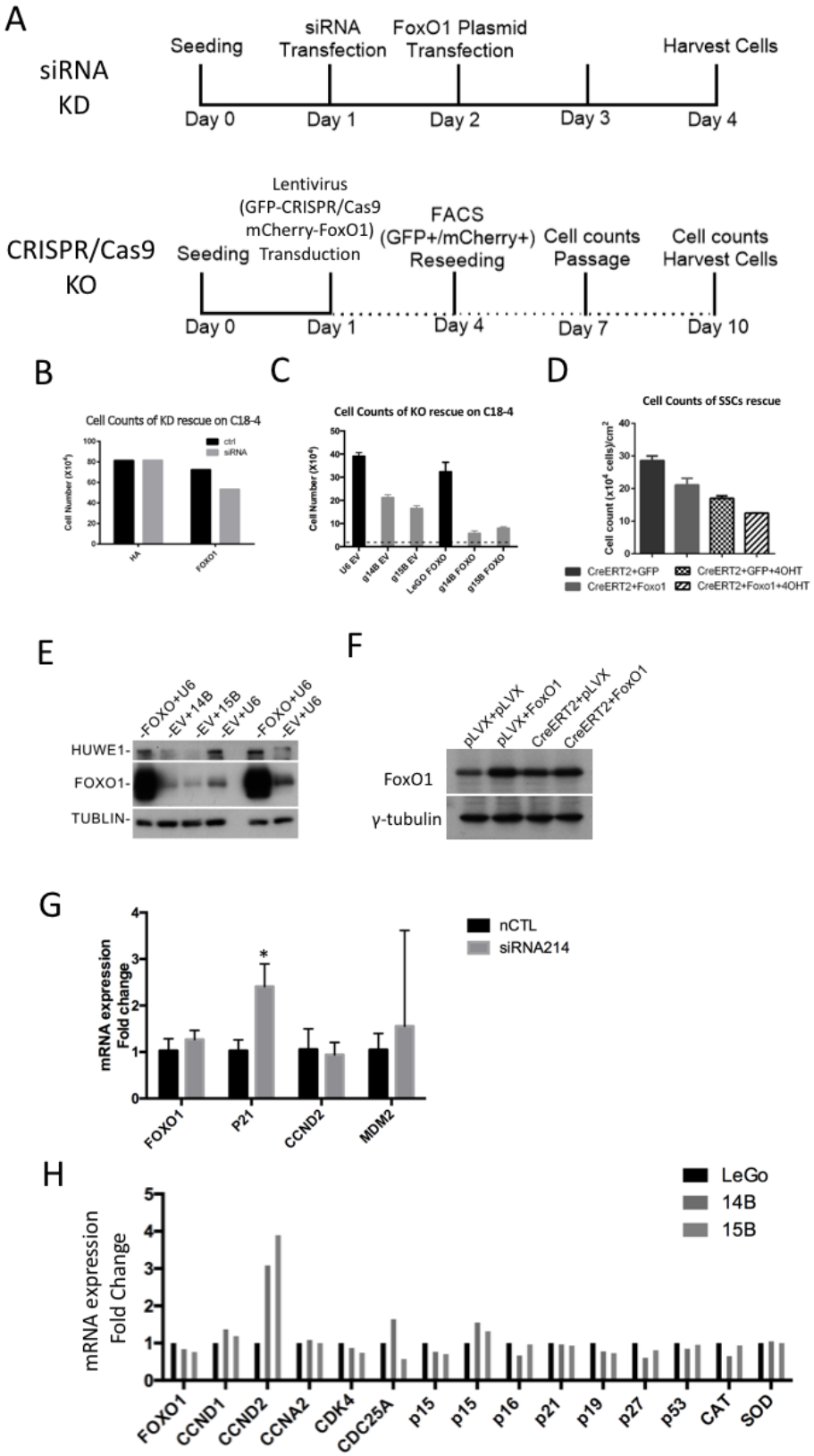
A
siRNA KD
Seeding
siRNA Transfection
FoxO1 Plasmid Transfection
Harvest Cells
Day 0
Day 1
Day 2
Day 3
Day 4
CRISPR/Cas9 KO
Seeding
Lentivirus (GFP-CRISPR/Cas9 mCherry-FoxO1) Transduction
FACS (GFP+/mCherry+) Reseeding
Cell counts Passage
Cell counts Harvest Cells
Day 0
Day 1
Day 4
Day 7
Day 10
B
Cell Counts of KD rescue on C18-4
ctrl
siRNA
Cell Number (X10⁴)
HA
FOXO1
C
Cell Counts of KO rescue on C18-4
Cell Number (X10⁴)
U6 EV
g14B EV
g15B EV
LeGO FOXO
g14B FOXO
g15B FOXO
D
Cell Counts of SSCs rescue
Cell count (x10⁴ cells)/cm²
CreERT2+GFP
CreERT2+Foxo1
CreERT2+GFP+4OHT
CreERT2+Foxo1+4OHT
E
-FOXO+U6
-EV+14B
-EV+15B
-EV+U6
-FOXO+U6
-EV+U6
HUWE1-
FOXO1-
TUBLIN-
F
pLVX+pLVX
pLVX+FoxO1
CreERT2+pLVX
CreERT2+FoxO1
FoxO1
γ-tubulin
G
nCTL
siRNA214
mRNA expression Fold change
*
FOXO1
P21
CCND2
MDM2
H
LeGo
14B
15B
mRNA expression Fold Change
FOXO1
CCND1
CCND2
CCNA2
CDK4
CDC25A
p15
p15
p16
p21
p19
p27
p53
CAT
SOD

Figure 9 Restoration of FoxO1 does not rescue cell number decrease phenotype.

A. Schematic outline of the rescue experiments by restoring FoxO1 upon Huwe1 KD or KO in the C18-4 cell line. B,C. Cell counts of indicated rescue experiments on C18-4. D. Cell counts of inducible Huwe1 KO SSCs with and without FoxO1 restoration. E. Reduced Huwe1 and overexpressed FoxO1 as shown by Western blotting, corresponding to KO-rescue experiment of Fig 3. C F. Restored FoxO1 level as shown by Western Blotting, corresponding to SSCs-rescue experiment of Fig 3E. G,H. Quantitative RT-PCR analysis of FoxO1 and well-known FoxO1 downstream targets from C18-4 cell KD and KO system.

Figure 10

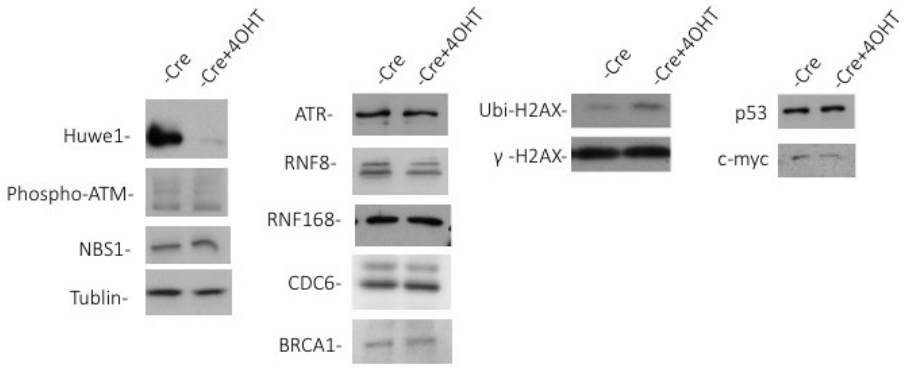

Figure 10 Huwe1 is inactivated in the primary SSC at the protein level. Inactivation of Huwe1 does not change of the level of γH2AX, pATM and other DDR pathway proteins as well as well-known substrates in SSC. Representative immunoblots from SSCs 3 days post tamoxifen treatment (4OHT) to induce Cre recombinase activity. γ-tubulin was used as loading control.

Figure 11

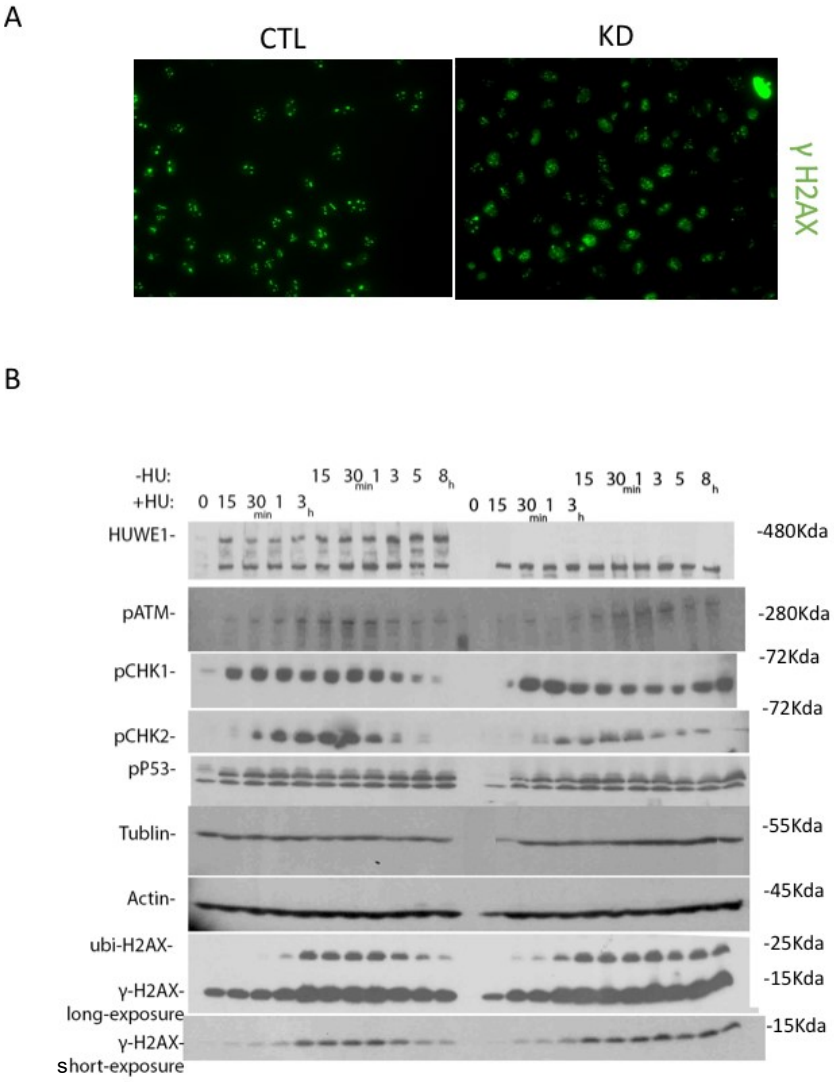

Figure 11 A. More C18-4 cells showing a greater number of γH2AX foci in Huwe1 depleted C18-4 cells. Representative immunofluorescence images of C18-4 cell 3 day post-transfection stained with antibodies against γH2AX (green). B. The activation (phosphorylation) of ATM at Serine 329 (pS329), CHK2 (pThr68), CHK1 (Ser345) and γH2AX was examined by Western blot at indicated time points after DSBs induction. Huwe1 depletion results in delayed and inhibited kinetics of DDR. Tubulin is a loading control. The numbers on the left indicate molecular weight in kDa. γ-tubulin was used as loading control.

TABLES

Table 1 Antibodies used in these studies

Protein name	**Company**	**Catalog #**	**Dilution for immunostaining**	**Dilution for Western blot**
Huwe1	Our laboratory	NA	1:100	1:500
FoxO1	Cell Signaling Technology	2880S	NA	1:1000
γH2Ax	Millipore	05-636	NA	1:4000
γH2Ax	Abcam	ab11174	1:2000	
H2AX	Bethyl	A300-082A	NA	1:5000
γtubulin	Sigma	T6657	NA	1:4000
ATM	Santa Cruz	sc-23921	NA	1:200
ATR	Santa Cruz	Sc-1887	NA	1:200
Phospho-ATM	Abcam	Ab36810	NA	1:1000
Phospho-ATR	Cell Signaling Technology	2853	NA	1:1000

BRCA1	Abcam	Ab191042	NA	1:1000
Phospho-CHK2	Cell Signaling Technology	2661	NA	1:1000
Phospho-CHK1	Cell Signaling Technology	2853	NA	1:1000
Phospho-P53	Cell Signaling Technology	9286	NA	1:1000
NBS1	Abcam	7860	NA	1:1000
P53BP1	Novubiso	7158	NA	1:2000
RNF168	Millipore	ABE367	NA	1:1000
Rad51	Santa Cruz	Sc-8349	NA	1:200
RNF8	Millipore	09-813	NA	1:1000
CDC6	Santa Cruz	sc-9964	NA	1:1000
P53	Abcam	Ab28	NA	1:2000
MYC	Abcam	Ab32072	NA	1:1000

Table 2 Oligonucleotides used in these studies

Gene name		Experiment	Sequence 5' - 3'	Type of Oligonuleotides
Huwe1 Exon 14 sgRNA	Top	CRISPR/Cas9 gene editing	CACCGCTTGTATAATGGGTTGATAG	DNA
	Bottom	CRISPR/Cas9 gene editing	AAACCTATCAACCCATTATACAAGC	DNA
Huwe1 Exon 15 sgRNA	Top	CRISPR/Cas9 gene editing	CACCGACTTGTGCGGAACTGCATTC	DNA
	Bottom	CRISPR/Cas9 gene editing	AAACGAATGCAGTTCCGCACAAGTC	DNA
Huwe1 siRNA 214	Sense	RNAi	ACACUGUUCUUAAGCUGCUACUAAA	RNA
Huwe1 siRNA 400	Sense	RNAi	CAGGATGACTATGATTGGCTGTGAT	RNA
Huwe1 siRNA 3519	Sense	RNAi	GGAGACAGATGGCTGCAAGAATT	RNA

Huwe1 siRNA 7536	Sense	RNAi	CGGAUCUGGGAACAGUACAAUUAUA	RNA

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