The Ovarian Requirement for Variant TFIID Subunit, TAF4b, in Mammalian Reproduction

by

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Lindsay A. Lovasco, Kimberly A. Seymour, Kathleen Zafra, Colin W. O'Brien, Christoph Schorl and Richard N. Freiman. Accelerated Ovarian Aging in the Absence of the Transcription Regulator TAF4b. *Biol. Reprod.* (in review).

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CONFERENCE ABSTRACTS AND PRESENTATIONS

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Lindsay A. Lovasco, Richard N. Freiman. Gametogenic Defects Lead to Sex-specific Levels of Infertility in TAF4b-null Mice, COBRE/INBRE Symposium, Cancer, Development, & Regenerative Medicine, May 2007.

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Lindsay A. Lovasco, Aron Gyuris, Ekaterina Voronina, Kenneth G. Geles, Michael T. Marr, Robert Tjian and Richard N. Freiman. Gonad-selective variants of Global Transcription Complexes in Mammalian Reproduction and Development, Gordon Research Conference, Mammalian Gametogenesis & Embryogenesis, June 2006.

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needed comic relief during the stressful times but also was a good friend to me when a laugh wasn't going to fix the situation. I will always be grateful to Steve, Katie and Viet, and each of them means more to me than I think they know.

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I would also like to thank my family who has always believed that I could do whatever I set out to do. I appreciate their tolerance of my crazy schedule and not always being able to visit as much as I'd liked. The fact that I know they are proud of what I do was a big motivating factor in why I continued during the toughest of times. Never once did they say "You're still in school?" even though I was coming up on the 23rd grade. I'd like to express my most sincere thanks to my mother who, despite enormous adversity, was able give my brother and me the tools we needed to become successful, independent adults. And while I have this little space to ramble, I'd just like to tell Tim how proud I am of him, too.

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While I am aware that this could be the longest acknowledgement section ever written, I still don't think it is enough to express how much I appreciate everyone who helped me to get here.

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Chapter 1

Introduction

General transcription factors and their variants

Transcription is an essential process to life and gives meaning to the genetic information stored within DNA. With increased organismal complexity comes the challenge of executing gene expression programs that are highly specific, yet readily adaptable to the changing needs of the cell. An additional consideration in higher order organisms is how to establish and maintain multiple cell and tissue types from an identical genomic template. To address these requirements, eukaryotes have evolved functionally diverse proteins to influence chromatin conformation, direct appropriate spatial and temporal gene expression and translate cellular signaling events into transcriptional output.

RNA Polymerase II and general transcription factors

RNA polymerase II (Pol II) is a large, multi-subunit complex that is responsible for mRNA synthesis of protein-encoding genes. In order to carry out transcription, Pol II works together with accessory proteins called general transcription factors (GTFs). Originally isolated as factors involved in the recruitment of Pol II to gene promoters, GTFs are typically multi-subunit complexes that work in a stepwise fashion to initiate basal transcription [1]. The GTFs were named for the fractions in which they were collected during the initial characterization, and are called, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH [2]. Pol II does not seek out DNA sequences itself but associates with a subset of the GTFs, TFIIA, TFIIB and TFIID, to recognize and define the start site of transcription, forming the pre-initiation complex (PIC) [3, 4].

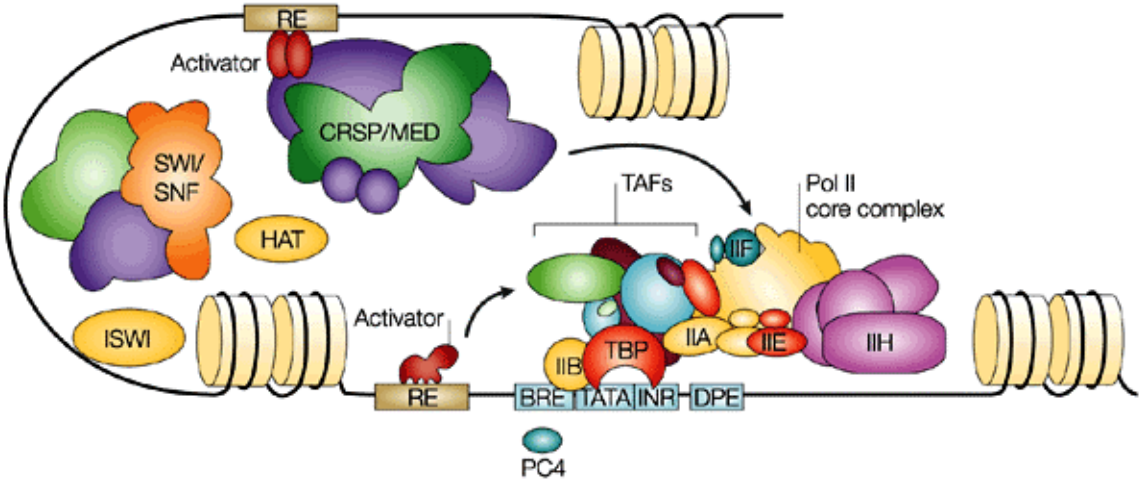


Figure 1. Eukaryotic pre-initiation complex at a RNA Polymerase II-transcribed promoter. Adapted from *Regulatory diversity among metazoan co-ativator complexes* [1].

Although the minimum requirement for Pol II transcription is the presence GTFs, the eukaryotic promoter can be a very crowded place, harboring not only the multi-subunit Pol II enzyme and GTFs, but activators, repressor, co-activators, chromatin remodeling factors, histone modifiers, and protein complexes that mediate interactions between all of them (Figure 1). Activated transcription is carried out by the concerted efforts of the factors present at the PIC at any given time, which varies by gene, cell type, and cell status.

Composed of the TATA-box binding protein (TBP) and approximately 14 TBP-associated factors (TAFs), TFIID is a critical component of eukaryotic transcription. TAF4 is a particularly important protein in this complex, as it is required for the physical integrity of TFIID, and has been shown to interact with the activator proteins Sp1 and CREB [5-9]. Recently identified variant TFIID subunits are expressed in a more

restricted fashion than the canonical subunits giving additional specificity to activated gene expression.

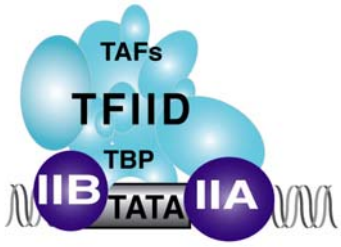
GTF variants in the germline

GTF germline variants of TFIID and TFIIA have been shown to be essential for a number of processes involved in reproduction (Figure 2). The first discovery of a GTF subunit variant was the TBP paralog, TBP-related factor 1 (TRF1), in *Drosophila* [10]. TRF1 has diverse functions and is involved in regulating both Pol II and Pol III transcribed genes. Although there is no vertebrate homolog of TRF1, several GTF variants have been identified in numerous species. Interestingly, the majority of GTF variants that have been identified are components of TFIIA or TFIID and have restricted gonadal expression in both *Drosophila* and in vertebrates [11].

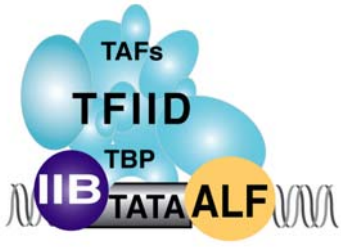
An alternate form of human TFIIA, TFIIA α/β-like factor (ALF; also called TFIIAτ), was the first testis-specific variant discovered [12-14]. ALF is able to completely replace TFIIA during gametogenesis in both mammals and frogs, demonstrating that these GTF variants are utilized in gonadal processes in distant vertebrate organisms [13, 15]. TFIID subunit variants of TBP and multiple TAFs have been identified in the gonads of species from *Drosophila* to human, and are in many instances restricted to the male germline. In mammals, a TBP variant called TBP-related factor 2 (TRF2 or TLF) is expressed in the testis and is required for spermiogenesis [16]. A class of TAF variants, called tTAFs, have specific testis expression in *Drosophila* and are required for functions such as meiotic progression and spermatid differentiation. tTAFs are distinct, paralogous genes of the more ubiquitously expressed dTAFs*; no hitter* (*nht*; dTAF4 paralog), *cannonball* (*can*; dTAF5 paralog), *meiosis I arrest* (*mia*; dTAF6

paralog), *spermatocyte arrest* (*sa*; dTAF8 paralog) and *ryan express* (*rye*; dTAF12 paralog) [17, 18]. The tTAFs are predicted to form a stable, alternate TFIID complex to promote expression of the genes involved in male fertility.

In mammals, gonadal TAF variants apparently do not form their own TFIID complex, but selected subunits will incorporate into the canonical complex to drive specialized transcription programs. In mammals, variants of TAF4 (4b), 5 (5L), 7 (7L) and 9 (9b) have been identified, all of which but TAF9b have confirmed, critical roles in fertility [19-23]. While TAF5L and 7L only affect male gonad function, TAF4b is required for normal fertility in both males and females [23-25]. Evolution of germline variant transcription regulatory proteins is not restricted to GTFs, but is also observed in the form of alternate histones that are expressed in the germline.

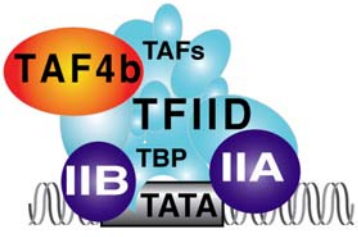


Canonical Pre-initation complex



Alternative TFIIA Complex

Alternative TBP Complex



Alternative TAF Complex

Figure 2. Alternative core promoter recognition factors in germ cell development. Adapted from *Specific variants of general transcription factors regulate germ cell development in diverse organisms* [11].

Study of gonadal-selective GTF variants addresses the fundamental question of how distinct cell and tissue types negotiate the genome to execute the gene expression programs that confer their identity. Understanding how these factors contribute to cell differentiation and development is an exciting area of transcriptional research that will increase not only our knowledge of how cell-type diversity is established, but also the biology of metazoan reproductive systems.

Generation and initial characterization of TAF4b-deficient mice

Identification of TAF4b

TFIID was originally thought to be a ubiquitous factor required for Pol II transcription but the discovery of tissue-enriched TFIID components introduced the idea of TFIID having additional, specialized roles. A screen for factors that associated with TFIID in a cell-type specific manner was conducted using co-immunoprecipitation with TBP from fractionated, TFIID-containing lysates. Of 8 human cell lines tested, the highly differentiated Daudi B (Burkitt Lymphoma) cell line contained a unique polypeptide that was barely detectable in the other cell lines [26]. This novel protein was determined to be a *bona fide* TFIID component in Daudi B cells and not a breakdown product of other TAFs. Subsequent cloning of this factor revealed that it shared a high degree of homology with the previously identified, ubiquitiously expressed TAF 4 (formerly, $TAF_{II}130$) and was named TAF4b (formerly, $TAF_{II}105$).

TAF4 and TAF4b domain homology and function

Between TAF4 and TAF4b, the highest degree of homology was found in the C-terminal domain, which contains a histone-fold motif, while the N-terminal domains were divergent. It had been shown that the N-terminal domain of TAF4 was involved in co-activation of gene transcription, and the initial characterization of TAF4b demonstrated that it, too, possessed co-activator functions. It is believed that incorporation of TAF4 and TAF4b into TFIID is not mutually exclusive and the level of TAF4b in Daudi B cells is sub-stoichiometric within TFIID. The structure of these TAFs is such that incorporation into the TFIID complex is carried out by the C-terminal domain, while the N-termial domain acts as a co-activator [27]. Because the N-termini of TAF4 and TAF4b are divergent, they may interact with different sets of activator proteins or have unique physical interaction properties, conferring additional specificity to TFIID-mediated transcription.

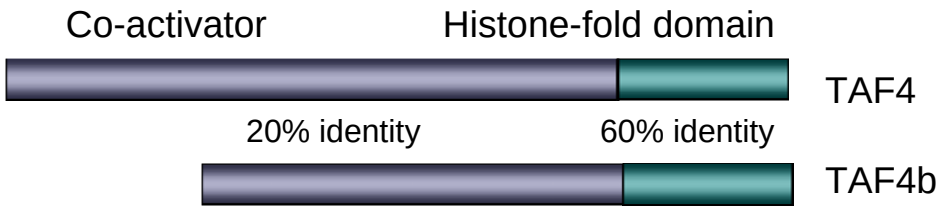


Figure 3. Domain identity between TAF4 and TAF4b. The N-terminal co-activator domain is divergent between TAF4 and TAF4b with only 20% amino acid identity, while the histone-fold domain is more conserved with 60% identity.

Female sterility observed in TAF4b-null female mice

To better understand the role of this protein *in vivo*, a TAF4b knockout mouse line was established using homologous recombination [23]. RNase protection assays revealed that TAF4b expression was detected mainly in the gonads and deletion of this gene did not interfere with viability. Offspring from TAF4b heterozygote crosses yield the expected Mendelian ratio of 1:2:1 [23]. Crosses using TAF4b-null mice revealed sex-specific fertility defects associated with loss of TAF4b [23, 24].

The first breeding trials of TAF4b-null mice indicated that males were fertile and females were infertile, prompting investigation as to the cause of female infertility. Many possible defects, stemming from multiple physiological processes, could lead to sterility in females. Since TAF4b is highly expressed in the ovary, it was likely that an ovarian defect was responsible, but deficits in mating behavior and uterine function needed to be ruled out. TAF4b-null females were verified to copulate with males, indicating that a behavioral problem was not responsible. To determine if a uterine defect was present, pseudo-pregnant TAF4b-null mice were implanted with 2-cell-staged embryos. Implantation and development of these embryos in TAF4b-null mothers occurred as in wildtype mothers, indicating that implantation and maintenance of pregnancy was not compromised, suggesting TAF4b-null defects arise in the ovary.

The sexually mature ovary is composed of follicles which are composed of a single germ cell, or oocyte, surrounded by somatic cells, called granulosa cells, which are supported by a basement of theca cells. The details of follicle growth, or folliculogenesis, will be discussed later.

At 12 weeks of age, the ovaries from TAF4b-null mice are about 50% smaller than their wildtype littermates. *In situ* hybridization revealed that TAF4b mRNA is most highly expressed in the granulosa cells of growing follicles, while TAF4 is present in multiple cell types including granulosa cells, theca cells and oocyte nuclei. If TAF4b were required for the granulosa cell function, as suggested by its mRNA expression, the infertility could arise from improper maturation and ovulation of oocytes, which are highly dependent on granulosa cells. Interpretation of data from assays aimed at determining the ovulation capacity of TAF4b-null mice is difficult. After breeding, eggs were collected from TAF4b-null mice with an average of 7 per animal, indicating that they are capable of ovulation. These eggs, however, did not undergo cleavage in culture, as healthy, fertilized eggs would. Although TAF4b-null mice could ovulate under natural conditions, super-ovulation regimens of administrated hormones on 2 month-old animals were not effective. While 120 oocytes were collected from 8 TAF4b heterozygotes, only 1 egg was isolated from the 8 TAF4b knockouts. Attempts to super-ovulate mice 5-6 months of age proved more successful with 13 mice producing 36 eggs but, still, these eggs did not cleave in culture. In a different study, it was found that immature TAF4b-null females ovulated about half the number of oocytes as wildtype following hormonal stimulation [28]. Together, these experiments show that TAF4b-null mice are capable of ovulation and oocytes can be fertilized but that the process is significantly impaired.

It is not clear to what extent ovulation is reduced or how the ability of TAF4b-null females respond to exogenously administered hormones is altered. To identify gene expression disruptions which contribute to TAF4b-null female phenotype of infertility, genome-wide gene expression was evaluated. The initial microarray was performed with

hormonally stimulated 8-week-and-older ovaries from wildtype and TAF4b-knockouts and showed that deletion of TAF4b reduces expression of activin, inhibin, cyclin D2, aromatase and follistatin, all of which are granulosa cell transcripts [29, 30]. A subsequent study showed that 15 day-old (unstimulated) or 21 day-old (hormonally stimulated) TAF4b-null ovaries expressed these genes at normal levels and could not identify any transcriptional mis-regulation using a candidate gene approach [28].

Although TAF4b target gene expression was still under debate, the physiological defects associated with loss of the transcription factor were quite clear. TAF4b-null females had compromised cumulus cell expansion both *in vivo* and *in vitro*. Cumulus-oocyte complexes (COCs) isolated after ovulation or induced to expand in culture show reduced number of granulosa cells that do not properly adhere to each other and the oocyte. Furthermore, TAF4b-null oocytes have a reduced capacity to complete meiosis I (46% vs 90% in wildtype), and although fertilization can occur in TAF4b-null oocytes, embryos cannot develop beyond the 2-cell stage [28].

Not only are defects observed at the end of oogenesis, but characterization of newborn TAF4b-null ovaries reveal that the pool from which the oocytes are recruited is defective from a very young age. Even though the number of oocytes present at birth is the same as wildtype, germ cell numbers are reduced to less than half those of heterozygous or wildtype littermates by postnatal day 3. Interestingly, this reduction does not influence recruitment into development because by postnatal day (pnd) 8, different TAF4b genotypes have equivalent numbers of primary follicles. A more in depth characterization of follicle recruitment revealed that although primordial follicle

numbers are reduced, primary follicle numbers are equivalent at 3, 6, and 12 weeks of age between wildtype and TAF4b-knockouts [25].

Another observation in TAF4b-null females is that they have elevated follicle stimulating hormone (FSH) levels from an early age. FSH is 2-3- fold higher in TAF4b-nulls than heterozygotes at pnd 23, and levels remain higher at subsequent time points taken of 26, 42, and 49 day-old mice [25, 28]. Elevated FSH is also observed in TAF4b-null males although it is not observed until 49 days of age [24]. Initially thought to be fertile, the TAF4b-null male is able to sire pups at 8 weeks but becomes completely infertile by 11 weeks of age. The male phenotype was not apparent in a mixed genetic background but was revealed upon back-crossing into a pure C57BL6 background. Like the female ovaries, testis size is greatly reduced by 12 weeks- a degeneration that appears to begin around 5 weeks of age [24]. The phenotype in TAF4b-null males appears to stem from an inability to maintain spermatogonial stem cells. In the testis, spermatogonial stem cells divide asymmetrically to renew themselves while producing cells that will ultimately undergo differentiation and meiosis to produce gametes. Unlike TAF4b-null females, the males had obvious transcriptional defects in key germline-expressed genes. At postnatal day 3 expression of spermatogonial stem cell markers *c-Ret*, *Plzf*, and *Stra8* is reduced. Interestingly, the genes mis-regulated in TAF4b-null males which are common to both ovaries and testes were not disrupted in the females, suggesting that TAF4b-target gene regulation has sex-specific features.

Mammalian meiosis in females

Primordial germ cells and early meiotic events during embryogenesis

During embryogenesis, primordial germ cells (PGCs) migrate from the epithelium of the yolk sac to the genital ridge which is located adjacent to the hindgut. Secretion of numerous cytokines and growth factors from both the presumptive genital ridge and PGCs themselves direct their movement [31, 32]. On arrival, the PGCs lose motility and begin to divide, signifying their transition to oogonia [33]. Mitosis proceeds until about 13.5 dpc (days post coitus) forming clusters, called germline cysts [34]. Oogonia do not complete cytokinesis and are connected to sister cells by intercellular bridges, allowing synchronous division [35]. During cyst development, oogonia interact with somatic cells in the ovary, which will become the pre-granulosa cells, and this entire unit organizes into ovarian cords which will remain as such until primordial follicle formation [36]. Between 13.5 and 15.5 dpc, oogonia begin to enter meiosis and are then referred to as oocytes [37]. There is evidence to suggest that meiotic entry proceeds in a wave from the anterior to the posterior of the ovary, which may have implications in the timing of follicle activation upon entry into puberty [37, 38]. Oocytes progress through the first stages of prophase I, arresting in diplotene. At this point, the oocytes have completed chromosome pairing, synapsis and crossing over. This process begins around 17.5 dpc, and by 5 days after birth, the majority of oocytes have reached diplotene [39]. Diplotene arrest can last until the end of reproductive lifespan, which in mice is 15 months (40-50 years in humans), and during this time sister chromatids remain connected at the centromere while the chromatin along chromatid arms decondenses and is actively transcribed [40, 41].

Primordial follicle formation

Concomitant with the final oocytes achieving meiotic arrest, the cysts begin breaking down to form primordial follicles, occuring between 2 and 4 days after birth [42]. During the same timeframe, a large fraction of oocytes die, and it is not completely understood why the organism expends energy to create these specialized cells only to destroy them. In humans, it is estimated that the oocyte pool is reduced by 80% during cyst breakdown [43]. One possible purpose for the elimination of germ cells is that oocyte death and cyst breakdown are coupled and fragmentation into smaller cysts requires death of oocytes within them for the formation of primordial follicles containing just one oocyte [37].

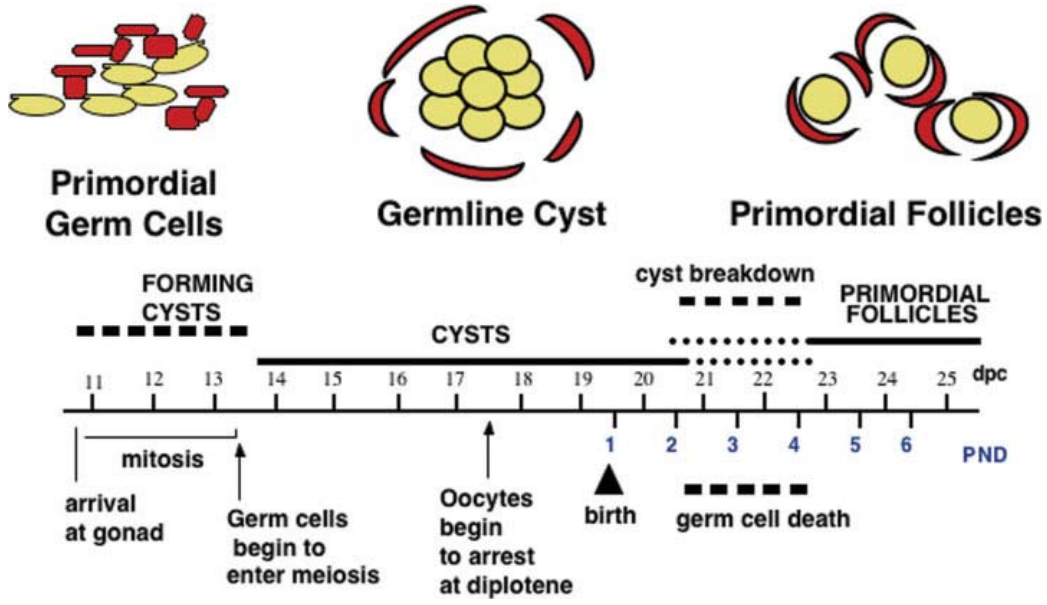


Figure 4. Timeline of female germ cell development in the mouse. Following arrival at the presumptive gonad, germ cells (yellow) undergo mitosis and begin to form germline cysts with somatic cells (red). Germ cells enter meiosis I at approximately 13.5 days post coitus (dpc) and arrest at the diplotene stage before birth. Between 2 and 4 postnatal days (pnd) of age, cyst breakdown leads to primordial follicle formation and marks a period of oocyte attrition. Adapted from *From primordial germ cell to primordial follicle:Mammalian female germ cell development* [35].

The mammalian phenomenon of germ cell attrition is similar to the programmed cell death observed in *Drosophila melanogaster* whereby one cell of the germline cyst is destined to become an oocyte while the other 15 serve as nurse cells, providing the oocyte with nutrients and organelles before their apoptosis [44]. Unlike *Drosophila*, mammalian oocytes do not participate in the transfer of bulk cytoplasm to a selected oocyte while sacrificing others, but parallels do exist and multiple alternate hypotheses have been proposed to explain the purpose of germ cell death during cyst breakdown. Similar to the phenomenon in *Drosophila* cysts, it has been proposed that mouse oocytes use the window of cyst breakdown to redistribute mitochondria [45]. Significant changes in the sub-cellular localization of mitochondria occurs just prior to cyst breakdown and it has been hypothesized that this provides a mechanism for sorting of the highest functioning mitochondria to the oocytes that will survive, and elimination of mitochondria with defective genomes by sending them to oocytes destined for apoptosis [45]. In line with preservation of the best mitochondria, the culling of oocytes during this stage could be a quality control mechanism to eliminate germ cells with chromosomal abnormalities or other defects that would compromise successful fertilization and development [45-47].

The synaptic and recombination events during meiotic prophase allow for the possibility of errors that may be detrimental, or even lethal. The timing of male and female meiosis is quite different which may contribute to the high degree of meiotic defects observed in oocytes, but not in sperm. Oocytes arrest in prophase of meiosis I (MI), as mentioned earlier, and remain in this state until they have matured fully and receive the ovulatory surge of LH, which triggers the completion of MI, extrusion of a

polar body, ovulation and second a meiotic arrest which persists until fertilization (Figure 5) [48, 49].

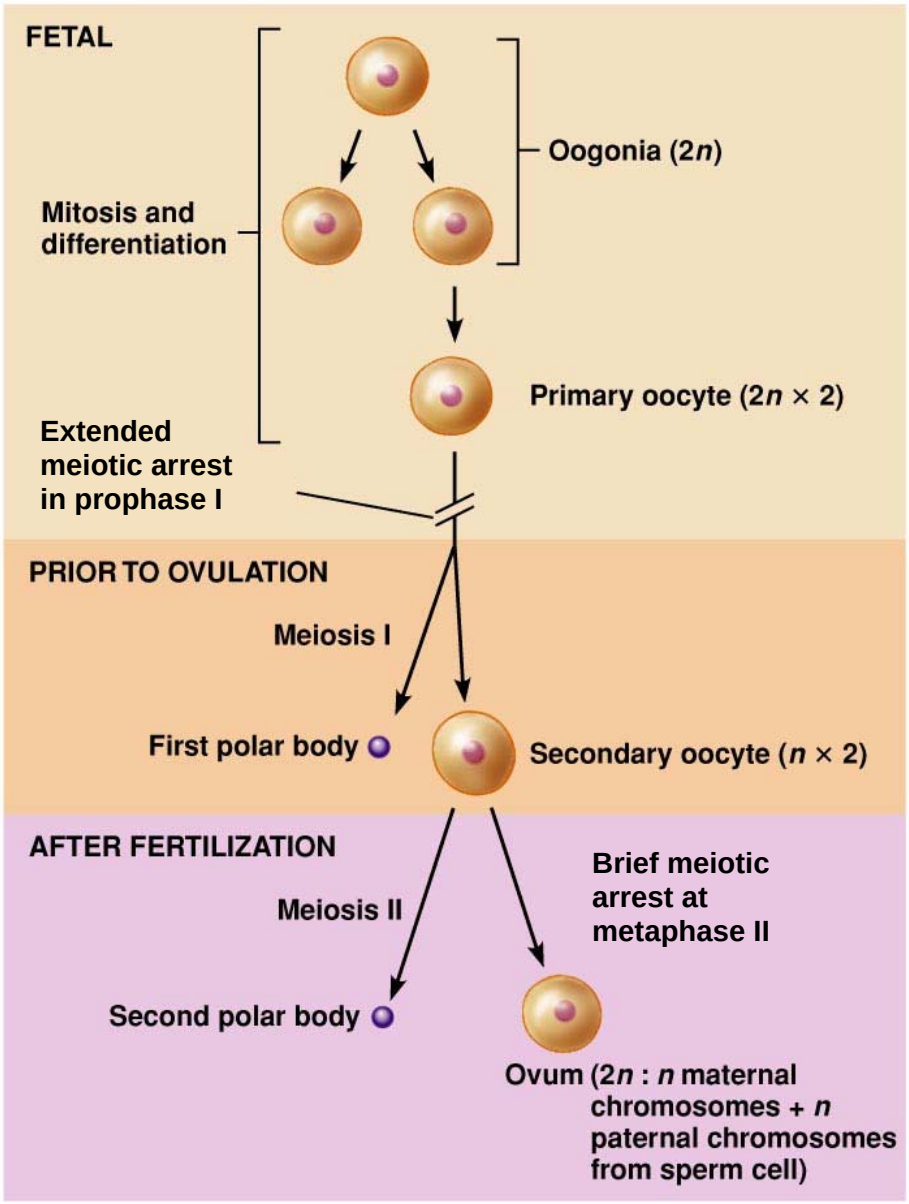


Figure 5. Meiosis in female mammals. Prior to birth, oogonia divide by mitosis, enter meiosis and arrest in prophase of meiosis I. After folliculogenesis, when the oocyte is fully grown, meiosis resumes and the first polar body is extruded. Following completion of meiosis I, the oocyte is arrested in metaphase of meiosis II until fertilization occurs, triggering the extrusion of a second polar body. Adapted from *Biology* [48].

Unlike females, males do not begin meiosis until puberty, at which point the entire process continues uninterrupted, taking about a week from the initiation of meiosis to the formation of sperm [50]. In humans, it is estimated that the rate of chromosomal abnormalities resulting from meiosis is about 20% in females, while only 3-4% in males. This discrepancy between sexes could be due to either more defects that occur in the female, or a lowered ability to either correct them or eliminate the cell, than in the male. Although the different timing of meiosis between males and females would support an argument for an increased occurrence of defects, this is hard to assay directly. On the other hand, there has been a great deal of data collected that points to sex-specific differences in addressing meiotic defects in the cell [50].

The majority of factors regulating meiosis in males and females is the same, however, targeted disruption leads to different phenotypic outcomes. In general, the inability to repair double stranded breaks, synaptic failure, and chromosome structural defects all appear to be causes for spermatocyte apoptosis. Disruptions of the same genes that regulate early meiotic events in females do not result in arrest; checkpoints monitoring chromosomal segregation are traversed [51]. Although oocyte maturation occurs in some cases, the resulting gametes are of poor quality, evidenced by cell death at later stages of oogenesis or increased aneuploidy following fertilization [50]. Figure 6 demonstrates the stages of meiosis I and developmental disparities between some male and female mouse knockouts.

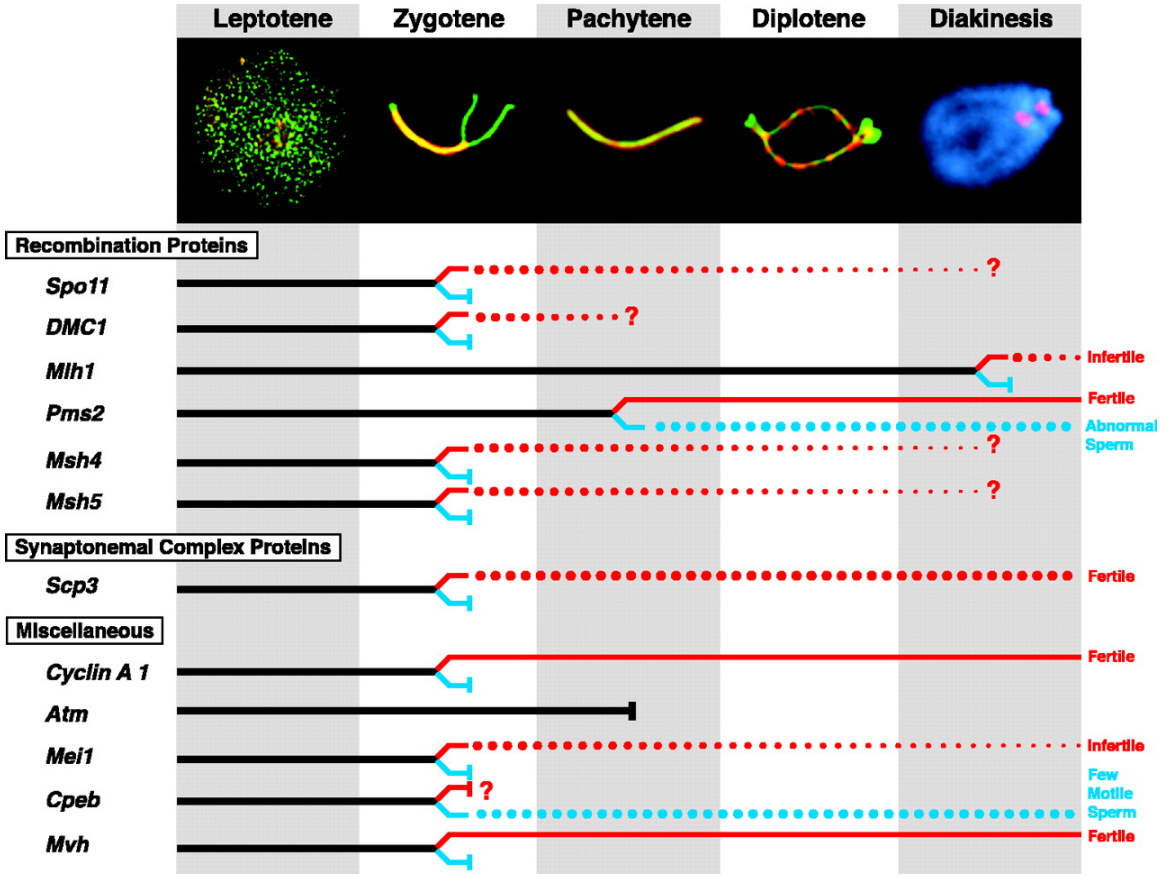


Figure 6. Male and female differences in meiotic progression and arrest points for mouse lines with targeted gene deletions. (Top) Stages of meiosis I prophase: presynapsis (leptotene), partial synapsis (zygotene), full synapsis (pachytene), desynapsis (diplotene), and chiasmate configuration (diakinesis). Components of synaptonemal complex: Green=Scp3, Red=Scp1. In the diakinesis panel, Blue=chromatin, red=kinetochore. (Bottom) Meiotic phenotypes for loss of indicated genes. Black lines indicate similar phenotypes between males in females, blue lines indicate male-specific phenotypes and red lines indicate female-specific phenotypes. Adapted from *Sex matters in meiosis* [50].

Folliculogenesis

Follicle growth

Once primordial follicles are established, they remain in a quiescent state until a portion of them is recruited into further development. Multiple activator and repressor pathways work together to prevent ectopic activation of the follicle pool, and how a small subset of these follicles is activated with each reproductive cycle is not well understood. One hypothesis states that oocytes are activated in the order in which they were established in the prenatal ovary [42]. Once activated, primordial follicles progress to the primary follicle stage, which is characterized by the transition of cell morphology changing from a single layer of flattened pre-granulosa cells to a layer of cuboidal granulosa cells surrounding an enlarging oocyte [52]. This transition does not occur rapidly, but is prolonged to allow the oocyte to gain size. The division of these granulosa cells to form a second layer marks the secondary follicle stage and is accompanied by formation of the zona pellucida (ZP). The ZP is a glycoprotein rich outer layer of the oocyte which is essential for oogenesis and subsequent fertilization [53]. The layers of granulosa cells continue to divide and interstitial cells are recruited to form a layer of theca cells around the follicle [54].

Up to this point, cellular proliferation has been gonadotropin independent and the most advanced follicle stage that can be achieved in this manner is termed ‘pre-antral’. Upon FSH stimulation, the follicle begins to form fluid-filled pockets within the layers of granulosa cells until they merge to form a single cavity, or antrum. During this time there is rapid granulosa cell proliferation. As the antrum becomes larger, the granulosa cells take on different characteristics depending on their location within the follicle. The

granulosa cells directly surrounding the oocyte are termed ‘cumulus,’ while the cells lining the outside of the follicle and are adjacent to the theca layer, are termed ‘mural’ [55].

Oocyte maturation

The final stage of folliculogenesis is known as ‘pre-ovulatory,’ in which the oocyte has completed maturation and is able to respond to pituitary signals (specifically luteinizing hormone (LH), which will be discussed in greater detail later). Throughout folliculogenesis, the oocyte grows in size from about 12 μm in the primordial follicle to over 70 μm in pre-ovulatory follicles, gaining volume to store RNA and organelles that will be used in embryogenesis, should fertilization occur [56]. Oocytes must achieve this level of growth to be able to re-initiate meiosis upon the LH surge [57]. Without LH receptors itself, the oocyte responds to LH signaling through changes in the surrounding granulosa cells, leading to breakdown of the oocyte nucleus (referred to as the germinal vesicle), asymmetric cell division, and the extrusion of a small unit called the polar body, signifying completion of MI. Not all oocytes in antral follicles will complete MI, though. Prior to germinal vesicle breakdown (GVBD), there is an important nuclear rearrangement that differentiates between the ultimate competence of the oocyte. Oocytes of antral follicles can be classified into either surrounded-nucleolus (SN), or non-surrounded-nucleolus (NSN) depending on the distribution of heterochromatin around the nucleolus. The default chromatin state in earlier staged oocytes is NSN and only those that undergo chromatin rearrangement to the SN conformation will be meiotically competent [58]. Switching to the SN state temporally coincides with transcriptional

silencing, which is maintained through the first zygotic cleavage, should fertilization occur [59-61].

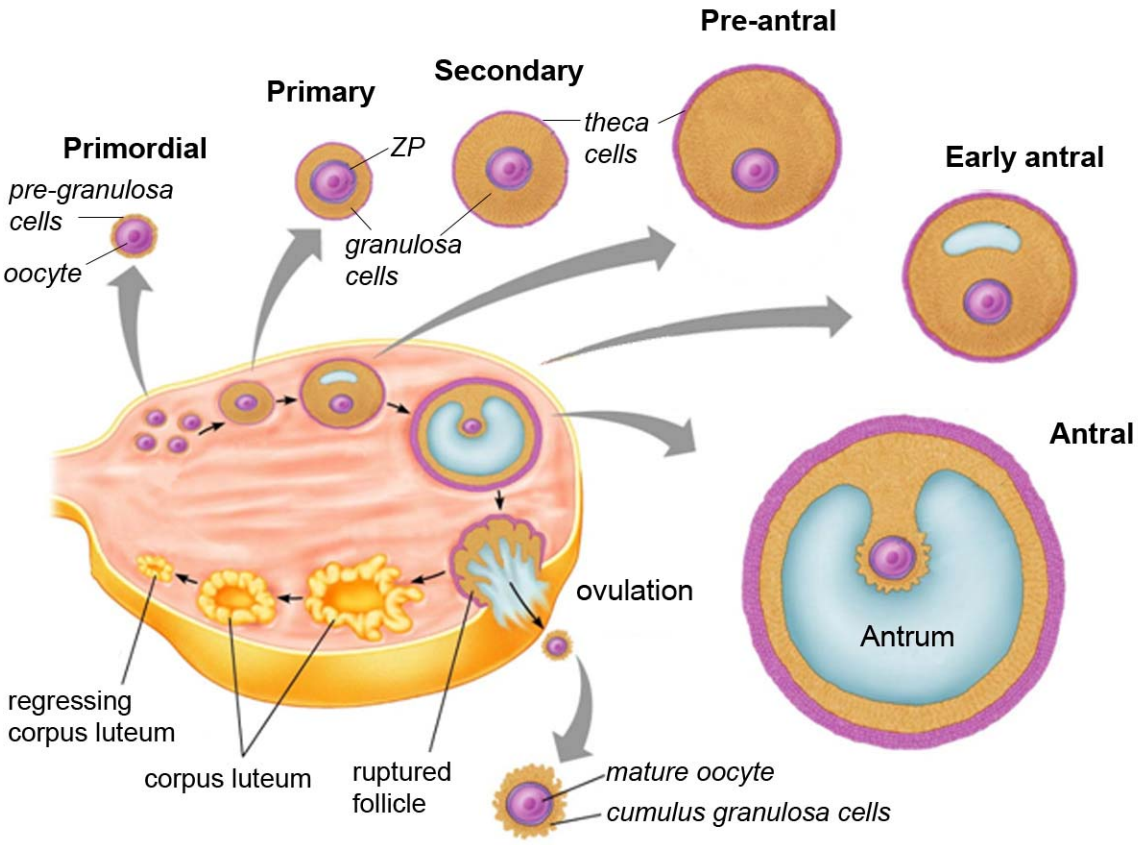


Figure 7. Mammalian folliculogenesis. Primordial follicles are composed of an oocyte surrounded by a layer of flat, pre-granulosa cells. Upon recruitment into development, the follicle transitions to a primary follicle marked by granulosa cells becoming cuboidal and the formation of the zona pellucida (ZP) around the oocyte. Granulosa cells divide to form additional layers, marking their entry to secondary follicles. They also recruit a layer of theca cells to the surround the follicle. As the follicle grows it develops a fluid filled cavity called antrum. At ovulation, mature follicles rupture and the cumulus-oocyte complex is released. The ruptured follicle differentiates into a corpus luteum which is then resorbed back into the ovary if pregnancy does not occur. Adapted from *Biology* [48].

Folliculogenesis ends with atresia or ovulation

The majority of oocytes recruited into development will not be ovulated, but undergo a programmed follicle death, termed atresia [40]. During folliculogenesis, atresia usually initiates in the granulosa cell layer, but in earlier staged follicles, oocyte death can initiate follicle death [62]. In follicles that escape atresia and survive to later stages, LH stimulation will trigger ovulation and the oocyte is released from the follicle, associated with its cumulus granulosa cells [63]. The mural granulosa and theca cells undergo differentiation into a structure called the corpus luteum (CL) [63]. If fertilization and implantation occurs, the CL is maintained and secretes progesterone to support pregnancy. If pregnancy is not achieved, the CL regresses and is resorbed back into the ovary [64]. After fertilization, the embryo relies on maternally deposited factors and does not robustly activate its own transcription on until the 2-cell stage [60, 61].

The length of folliculogenesis varies by species but for humans, the timing from follicle activation to ovulation is 85 days, while in rodents, the process takes approximately 14 days [65]. One complete hormonal cycle takes 28 days in the human and 4-5 days in the mouse. The development of a single oocyte spans multiple hormonal cycles, indicating that follicles are continually being activated and develop asynchronously despite receiving the same pituitary signals.

Hypothalamic-Pituitary-Ovarian axis

The molecular events that occur at each stage of folliculogenesis dictate cellular receptivity to hormonal stimuli and create an intricate balance of both pituitary and ovary-derived factors. The major hormonal components of the female reproductive

system are estrogen, follicle stimulating hormone (FSH), luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH). FSH and LH are referred to as peptide gonadotropins. They are glycoprotein dimers, each composed of an identical α subunit (Cga), and a unique β subunit (Fshb, Lhb), and are produced in the pituitary [66]. GnRH is released from the hypothalamus in a pulsatile manner, and the frequency of these pulses influences the subsequent release of FSH and LH from the pituitary [67]. Estrogen is a steroid hormone that is synthesized in the ovary in its most potent form, 17-β-estradiol (E2) [68]. The levels of these hormones predictably rise and fall throughout the estrous cycle, (the mouse equivalent of the human menstrual cycle), in response to the concerted efforts of positive and negative feedback in the hypothalamic-pituitary-ovarian axis (Figure 8). By altering somatic-cell hormonal sensitivity, controlling their own expression, and influencing gonadal gene expression, endocrine factors are key components in the regulation of follicle development and ovulation.

Estrogen is first produced by ovarian follicles in the late pre-antral stage [69, 70]. Estrogen biosynthesis relies on both theca and granulosa cell processes [71, 72]. Androgens, predominantly in the form of androstenedione and testosterone, are produced in the theca cells upon LH-induced conversion of cholesterol [73]. In the granulosa cells of antral and later staged-follicles androgens are converted to estrogen by the granulosa-cell-specific enzyme, aromatase [74]. The amount of estrogen produced is positively correlated with the size of the follicle, with the largest, pre-ovulatory follicles producing the most [69]. Estrogen feeds back positively to estrogen producing granulosa cells thereby amplifying its own synthesis [75].

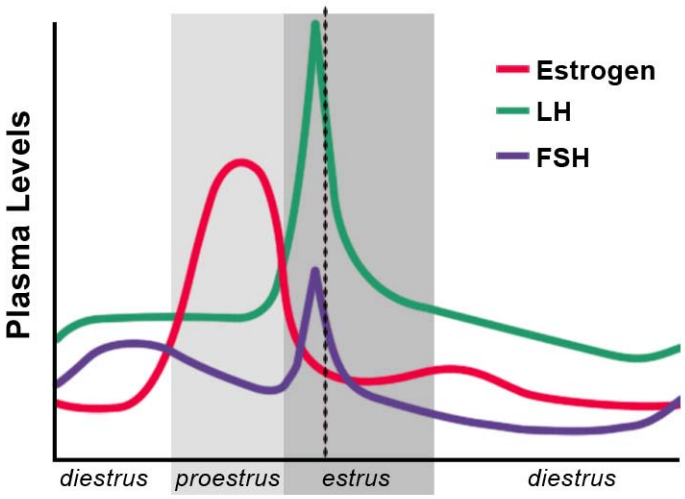


Figure 8. Estrous cycle in the mouse. Hormone levels are low during the state of diestrus. Rising estrogen signifies proestrus, followed by a surge of FSH and LH during estrus. Ovulation is demarcated by the dotted line.

Ovarian feedback mechanisms

Feedback signaling to the pituitary is influenced by the level of circulating estrogen. Before large pre-ovulatory follicles are present, estrogen is produced at very low levels in the ovary. Low estrogen levels signal to the hypothalamus keeping GnRH low, and preventing LH and FSH production and secretion from the pituitary. At the same time, estrogen sensitizes the gonadotropin producing cells, or gonadotropes, so that when GnRH is present, they respond strongly. Once the ovary contains large follicles that cause high levels of circulating estrogen, inhibition of GnRH is relinquished, leading to release of GnRH from the hypothalamus followed by FSH and LH release from the pituitary. This is known as the estrogen-induced gonadotrophin surge [EIGS], during which LH drives ovulation and initiates lutenization of theca and granulosa cells [76]. To bring gonadotropin levels back down after the surge, FSH exerts a negative feedback on the hypothalamus, decreasing GnRH secretion. Inhibin, a granulosa cell product, is also involved in reducing FSH levels (Figure 9).

Hormones are sensed by the cell through cognate receptors. The GnRH receptor (GnRHR), FSHR and LHR all belong to the seven-transmembrane G-protein coupled receptor family [77, 78]. GnRH is received by gonadotropes and activates a variety of G-proteins to turn on multiple pathways, the most prominent of which involve Ca^{2+}, PKC, ERK, JNK, p38, and c-Src [79]. GnRH is released in pulses from the hypothalamus and stimulates the expression and secretion of LH and FSH. The frequency of GnRH pulses determines if LH or FSH will be produced in the gonadotrope [80]. Low frequency pulses of GnRH, at about 1 every 120 minutes, will favor FSH production, while high frequency pulses, 1 every 10 minutes, stimulate LH production [79]. It is believed that estrogen feedback controls hypothalamic GnRH production and/or release, and during the earlier stages of folliculogenesis, estrogen concentration is low, and GnRH pulses are infrequent, allowing for preferential FSH production. As follicles near maturation, they secrete more estradiol, which increases GnRH pulse frequency, causing preferential LH production from the pituitary, which in turn causes a surge of LH, inducing ovulation. This is a very interesting phenomenon, and it is not well understood how pulse frequency translates to selective gonadotropin production.

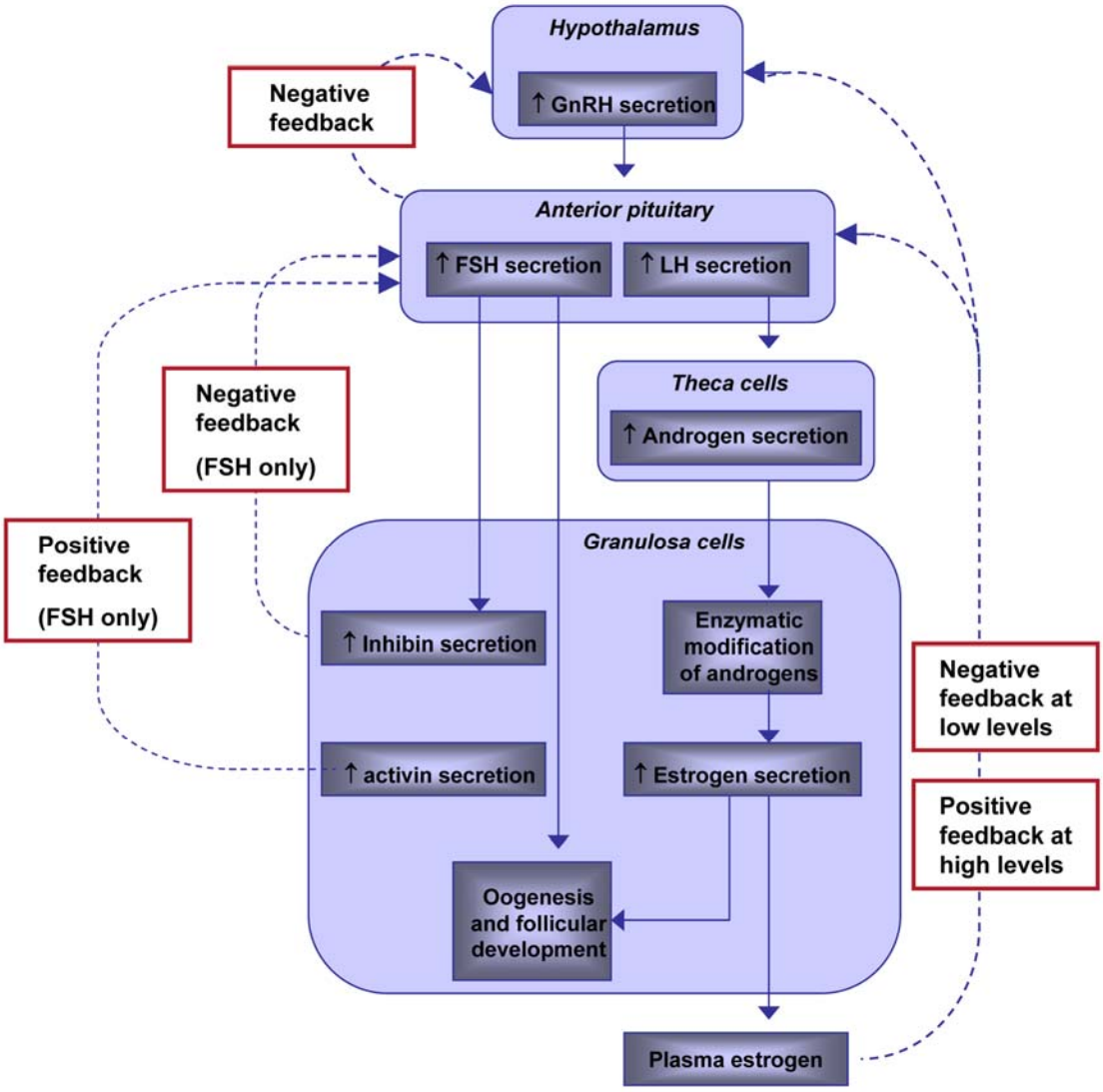


Figure 9. **Hypothalamic-pituitary-ovarian axis.** GnRH from the hypothalamus controls FSH and LH production and secretion from the anterior pituitary. FSH acts on granulosa cells to promote inhibin production and follicular development. LH acts on theca cells to increase androgen production. Androgens are converted to estrogen within the granulosa cell. Circulating inhibin feeds back to the pituitary to inhibit FSH production. Low levels of estrogen are inhibitory, but high levels of estrogen feeds back to the hypothalamus and the pituitary to promote gonadotropin production and release. Adapted from *Biology* [48].

Gonadotropin-mediated effects on the ovary

FSH receptors on granulosa cells receive signals from the pituitary. FSHR is present starting at the primary follicle stage, increases in abundance as the follicle grows, and is then greatly reduced following ovulation and corpus luteum formation. It is the follicle's ability to respond to FSH that determines its ultimate fate- ovulation or atresia. The early stages of follicle development do not require FSH, but to progress to the antral stage, the granulosa cells must be receptive to FSH. To allow for this, follicles express more FSHRs as they grow. Later in the cycle, when FSH levels fall, follicles that are highly sensitive to FSH will survive, establishing themselves as dominant follicles, while smaller follicles with less FSHR will undergo atresia.

LHR is found in theca cells of advanced-staged follicles where it receives LH signals and regulates androgen production. LHR is also found in the follicle cells following ovulation, luteinized granulosa or luteal cells, where it regulates progesterone production. Follicles become receptive to LH upon induction of the LHR by FSH, so only the follicles that have sufficiently developed will respond to the LH surge by ovulating [81]. If ovulation results in pregnancy, LHR expression is maintained in the corpus luteum (CL), but if pregnancy does not occur, the CL regresses and LHR is no longer expressed [82]. Activation of FSHR and LHR raises intracellular levels of cAMP and activates Protein Kinase A (PKA) [83]. Little is known about the transcriptional regulators that mediate cellular responses to these gonadotropins.

Estrogen-mediated effects on the ovary

Estrogen signaling occurs much more differently than gonadotropin signaling. Within the ovary there are two distinct estrogen receptors, ERα and ERβ [84]. These nuclear

receptors are composed of the same domains; AF-1, an activation domain located at the N-terminal domain, a centrally located DNA-binding domain (DBD), and a ligand dependent activation domain, AF-2, which is located in the ligand binding domain (LBD) of the C-terminus [85]. When estrogen is present, its binding to ER initiates a transcriptional response which is highly context dependent; the outcome is dependent on the other co-regulatory proteins present in that cell at that time. Ligand-bound ER can bind directly to estrogen response elements in target gene promoters or can interact with other transcription factor complexes such as Fos/Jun or SP-1 to influence transcription of genes without EREs [86, 87]. ER expression is heterogeneous within the ovary. Granulosa cells are the primary location of ERβ expression and estrogen biosynthesis. In contrast, ERα is largely expressed in theca and interstitial cells, which synthesize androgen and androgen precursors [88]. Using transgenic ER mouse knockout models, it was determined that the main ovarian role of ERα is to regulate feedback of gonadotropins, while ERβ is critical for granulosa cell function and differentiation [89].

Activin, inhibin, follistatin

Maintaining the correct balance of circulating hormones and receptor expression is critical for normal fertility. In addition to the self-regulation of hormone and receptor synthesis, other ovary- and pituitary-derived factors play an important part in controlling hormone action. The proteins activin, inhibin, and follistatin are key components of the reproductive endocrine system.

Activins and inhibins are members of the TGF-β superfamily of proteins which is involved in signaling and regulation of numerous physiological processes. Biologically active as dimers, inhibins are composed of one α subunit, encoded by inhibin α (inha),

and either a βA or a βB subunit, encoded by inhba or inhbb, to form inhibin A (α:βA) or inhibin B (α:βB), respectively (Figure 10). The β subunits can homo- or hetero-dimerize to form activin A (βA: βA), activin B (βB: βB) and activin AB (βA: βB). The subunit composition of the β subunits are highly similar with 63% sequence identity, but they have distinct roles and are differentially regulated during development and in postnatal life [90].

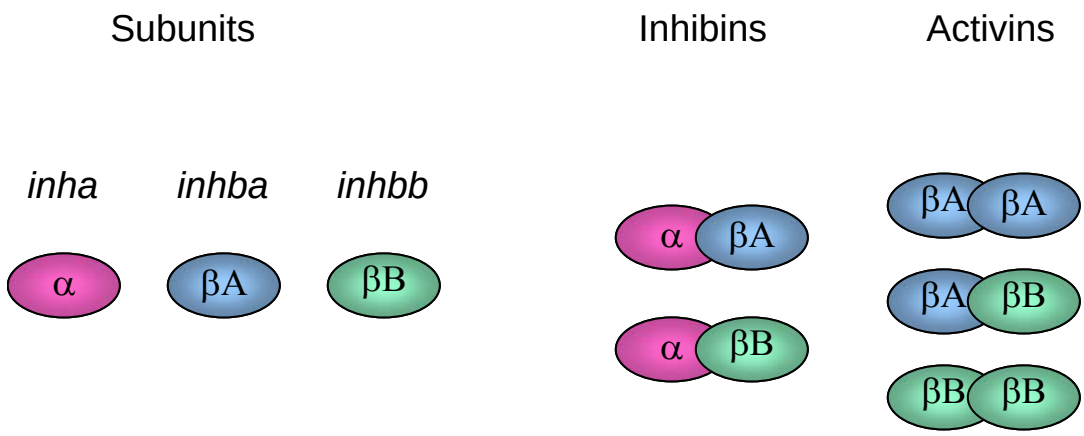


Figure 10. Activin and inhibin molecules. Inhibins and activins are composed of shared subunits from *inha, inhba and inhbb* gene products.

Using mouse-knockout models to identify the individual roles of βA and βB, it was discovered that βA disruption results in neonatal lethality, while mice null for βB are viable but exhibit multiple defects including problems with eyelid closure and birthing/nursing problems [91, 92]. While these proteins have important roles in embryogenesis and other postnatal biological processes, most of the research currently being conducted on activin and inhibin is focused on their roles in gonadal regulation. The main source of activin and inhibin synthesis in females is the granulosa cell, with lower levels of expression in the pituitary. The ultimate effects of activins and inhibins

oppose each other, with activins promoting FSH action and inhibins reducing FSH action by regulating both FSH and its receptor [90].

Although there is some evidence of inhibin in autocrine signaling in granulosa cells, inhibins are mainly secreted into the blood stream and act on the pituitary, which in turn, influences the ovary [93]. Activin is not readily detected in blood serum and acts primarily in an autocrine/paracrine manner [94]. Binding of activin to extracellular receptors on granulosa cells upregulates FSHR, and binding of pituitary-produced activin to gonadotropes increases FSHβ synthesis [90]. Inhibins bind to both their cognate receptor, betaglycan, and activin receptors, ACRIIA and ACRIIB2, which prevents activin binding [90, 95]. Of the two types of activin receptors, inhibin B has up to 4 fold higher affinity for ACRIIB2 than inhibin A, which has a higher affinity for ACRIIA [96, 97]. The ultimate cellular response to activins and/or inhibins depends on the relative abundance of activin and inhibin subtypes, along with the types of receptors present in a particular cell.

Ascertaining the individual functions of inhibins or activins is quite difficult due to their shared subunit composition, but it appears that activins are involved in promoting granulosa cell proliferation. Using *inha* knockout mice, which lack the α subunit, Matzuk *et al.* report that loss of inhibins cause uncontrolled granulosa cell proliferation and ovarian tumor development [98]. In transgenic mice deficient for ACRIIB2, follicles are arrested in the early antral stage [99]. Cultured pre-antral follicles secrete activin A, and exogenous activin A treatment induces granulosa cell proliferation *in vivo,* which can be inhibited by follistatin, further demonstrating a role for activins in proliferation [100, 101].

Inhibin β subunit expression is dynamic throughout the reproductive cycle as follicles develop. βA subunit expression remains fairly constant in granulosa cells of all follicular stages, while βB expression is highest in antral follicles. While the α subunit has been documented at all follicle stages, the increase in βB before ovulation represents a switch from inhibin A (α:βA) to inhibin B (α:βB) in the advanced-stage follicle, suggesting a role for inhibins in follicle selection [90, 102]. Although conflicting data make interpretation difficult, generally, the activin:inhibin ratio is higher in small follicles, suggesting that not only is there a switch in inhibin subtypes as follicles grow, but also a switch from activin to inhibin as the predominating subunit arrangement [103].

Another protein that opposes activin action, thus promoting FSH suppression, is follistatin [104]. Transcribed in both the ovary and pituitary, follistatin binds specifically and strongly to activin thereby irreversibly inactivating it. The affinity of follistatin binding to activin is at least as much as activin's affinity to its receptor, so once bound by follistatin, activin can not act on its receptor. Follistatin is most highly expressed in the ovary, and within the ovary it is most highly expressed in the granulosa cells of pre-antral and antral follicles, with much lower expression in primordial and ovulatory follicles [105]. It is believed that the main role of follistatin in the ovary is to reduce the bioavailability of activin [106].

The functions of follistatin, activins and inhibin must be evaluated with respect to one another. Taken together, the data show that these factors regulate folliculogenesis through autocrine, paracrine and endocrine signaling in, and between, the ovary and pituitary. Generally, as follicles grow, there is a shift from excess activin to low levels of free activin, and higher levels of inhibin and follistatin, which co-incides with

gonadotropin-independent to gonadotropin dependent follicle growth. Because granulosa cell expression levels of follistatin, inhibin and activin change with follicular stage, there is also a possible role for the balance of these factors in dominant follicle selection. Regulation of these factors in the pituitary is also a means of ensuring the timely release of FSH. Correct expression of follistatin, inhibin and activin creates a delicate balance that is critical to progress through folliculogenesis and produce a healthy, mature oocyte.

Premature ovarian failure

Cessation of menses, or menopause in women, signifies the depletion of ovarian follicles and the end of reproductive lifespan. The mean age for menopause in women in 50±4 years, and menopause occurring before 40 years of age is considered premature [107]. Approximately 1% of the female population experiences this fertility defect, which is clinically diagnosed as premature ovarian failure (POF) or the currently preferred term, primary ovarian insufficiency (POI). Although the affected women experience the same high FSH levels observed in menopausal women, POI differs in that there is varying and intermittent ovarian function in about 50% of cases and about 5-10% of patients can conceive a child without treatment. Although several genes have been found disrupted in women with POI, about 90% of the cases cannot be attributed to known genetic causes [108].

The two main underlying causes of POI are ovarian follicle dysfunction and follicle depletion. In the case of follicle dysfunction, oocytes are present in the ovary but are prevented from proper maturation due to one or multiple defects. Follicle depletion represents the case in which an inadequate, or absent, primordial follicle pool results

from failure at one or more developmental steps; establishment, accelerated expenditure of follicles, or autoimmune or toxicant-induced destruction of follicles [108]. Usually, the onset of puberty and menses is normal and women only seek medical attention after experiencing irregular or absent menstrual cycles, or upon unsuccessful conception attempts.

The majority of POI cases are believed to arise sporadically but a study of the family histories in 160 patients with idiopathic POI indicated familial link in about 30% of them, indicating that there may be underlying genetic causes that have not yet been characterized [109]. Known, heritable syndromes are associated with fertility defects and account for about 10-15% of patients with POI. A number of disruptions in single genes have been identified in cases of POI without non-reproductive health problems. Mutations in FSHβ, FSHR, LHRβ, LHR BMP15, INHA, DIAPH2, have been documented, but they are all very rare and often only documented in isolated geographic locations [108, 110]. The known causes of POI in women are listed in Table 1.

Turner syndrome affects 1 in 2500 females and manifests with congenital lymphoedema, short stature and ovarian failure. About 50% of patients are missing an entire X chromosome (45,X), while in 5-10% of patients, the long arm of the second X chromosome is present (46X, i(Xq)), and the remainder of patients have chromosomal mosaicism, such as 45,X/46XX [111]. In general, women with Turner syndrome had normal numbers of oocytes until the 3rd month of fetal life, at which point apoptosis was accelerated resulting in oocyte depletion by the time they were 10 years of age. Only 10% of women missing an entire X chromosome achieve menarche, while women who have mosaic karyotypes are more likely to menstruate and may actually cycle normally for a

number of years before the onset of POI. The molecular cause for infertility in Turner syndrome patients is not well established, and it has been proposed that improper pairing of the X chromosome could result in failure to traverse a meiotic checkpoint, leading to oocyte apoptosis, or there may be critical genes on the X chromosome that are required for ovarian function.

X chromosome deletions, inversions and balanced X:autosome translocations are the most common genetic causes of POI [112]. Mapping of these mutations has yielded surprisingly few target genes to attribute the observed infertility. One patient exhibited a breakpoint mutation that disrupted diaphanous (DIAPH2), which had previously been characterized in flies as a gene required for oogenesis and spermatogenesis [113-115]. Because so few single genes have been found to be disrupted in X-linked ovarian failure, it is postulated that dosage compensation may be altered or that apoptosis is initiated after a failed checkpoint [116, 117].

Fragile X premutations have also been linked to POI. In patients with Fragile X syndrome, there are more than 200 CGG repeats in the 5' UTR of the FMR1 gene, leading to mental retardation and autism. The normal number of repeats in healthy individuals is fewer than 40, but mothers of children with Fragile X syndrome have between 55 and 200, and upon transmission to her fetus, the premutation expands to a full mutation, shutting off transcription of the FMR1 gene [118]. 13-26% of female carriers of the premutation experience POI, and the age of fertility loss is inversely correlated with number of repeats [119]. Interestingly, women with the full mutation do not experience fertility problems, suggesting that there is a gain of function toxicity due to increased expression of FMR1 mRNA in carriers [120]. With the majority of POI

occurring with no known cause, it is of great medical interest to understand the normal process of ovarian aging and how its perturbation could lead to early infertility in women.

Table 1. Causes of premature ovarian failure. Adapted from *Premature ovarian insufficiency: a more accurate term for premature ovarian failure* [117].

Causes	Gene identification	Gene location
Genetic		
X chromosome		
X chromosome monosomy		
45X/46XX mosaic		
X chromosome translocations or partial deletions		
BMP15	Bone morphogenetic protein 15	Xp11·2
DIAPH2	Diaphanous	Xq22
FMR1 premutation	Fragile X mental retardation protein 1	Xq27·3
FMR2	Fragile site, X-linked, E	Xq28
Autosomal genes		
INHA	Inhibin α subunit	2q33-q36
FOXL2	Forkhead transcription factor	3q23
GALT	Galactose-1-phosphate uridyl transferase	9p13
ATM	Ataxia telangectasia mutated	11q22·3
POLG	Polymerase (DNA directed) γ	15q25
BLM	RecQ protein-like-3 DNA helicase	15q26·1
AIRE	Autoimmune regulator polyglandular failure type 1	21q22·3
EIF2B-2	Eukaryotic translation initiation factor 2B, subunit 2	14q24
EIF2B-4	Eukaryotic translation initiation factor 2B, subunit 4	2p23
EIF2B-5	Eukaryotic translation initiation factor 2B, subunit 5	3q27
Autosomal genes affecting follicle function		
FSHR	FSH receptor	2p21-p16
LHCGR	Luteinizing hormone/choriogonadotrophin receptor	2p21
STAR	Steroidogenic acute regulatory protein	8p11·2
CYP17A1	17-α hydroxylase/17,20-lyase enzyme	10q24·3
Follicle destruction		
Autoimmune		
AIRE	Autoimmune regulator polyglandular failure type 1 or APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy)	21q22·3
HLA association	Polyglandular failure type 2	
Iatrogenic		
Surgery		
Chemotherapy		
Radiation therapy		

Research Aims

This thesis aims to examine TAF4b's contribution to diversification of mammalian gene expression. The majority of the work presented here focuses on characterizing ovarian defects caused by deletion of TAF4b in female mice. Divergence of TAF4b from its paralog, TAF4, has lead to novel transcriptional roles for TFIID in mammals, the most apparent of which are observed in reproduction. We used the mouse ovary and gonadally-derived mammalian cell lines to uncover non-redundant roles for TAF4b. We also examined how TAF4b contributes to gene expression in systems outside of the ovary. This work aims to examine:

1) TAF4b-dependent processes in mammalian granulosa cells
2) TAF4b-dependent processes in mammalian oocytes
3) TAF4b expression and regulation in somatic tissues

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Chapter 2

Granulosa cell survival and proliferation are compromised in TAF4b-null ovaries

Abstract

Mammalian oocyte development requires productive interactions between somatic granulosa cells and a single germ cell within the ovarian follicle. Granulosa cells provide essential nutrients to the oocyte to sustain its growth throughout folliculogenesis, and the two cell types engage in paracrine and autocrine signaling to coordinate the events of oocyte maturation. TAF4b is a gonadal-enriched variant of the TFIID component, TAF4, that is essential for female fertility in the mouse and its mRNA expression patterns suggest that it may be functioning in granulosa cells. To determine the etiology underlying female infertility in TAF4b-null mice, we have examined its role in granulosa cell transcription and characterized multiple disruptions in granulosa cell processes. Here we report that TAF4b protein is highly enriched in granulosa cells and that without TAF4b, granulosa cells exhibit decreased proliferative capacity in response to estrogen and follicle stimulating hormone (FSH) and increased apoptosis. While increased apoptosis indicates a defect in granulosa cell survival, normal levels of granulosa cell survival factors in the Akt, Foxo and Pten pathways are present in 4 week-old, but not 16 week-old TAF4b-null ovaries. At 16 weeks of age, there is depletion of granulosa cell Foxo proteins as well as a decrease in the phosphorylated form of Foxo3a. siRNA-mediated knockdown of TAF4b and TAF4 in cultured NT-1 granulosa cells confirm c-Jun, cyclin D2, inhibin βA and Igfbp-3 as TAF4b target genes, but also implicate a role for TAF4 in the expression of c-Jun, cyclin D2, and Igfbp-3. While a number of granulosa cell processes are perturbed, granulosa gene expression disruption resulting from loss of TAF4b may only cause a portion of ovarian defects observed in the TAF4b-null mouse.

Introduction

The mammalian ovary is composed of both germ-line and somatic cells, both of which are equally important to female fertility. The main functions of the follicle are to generate a fertilizable oocyte and to produce and respond to hormone signaling between the ovary and pituitary. The oocyte and somatic granulosa cells are functionally inter-dependent and disruption of genes specific to either cell type can lead to infertile or sub-fertile phenotypes.

TAF4b is a gonadal-enriched component of the TFIID general transcription complex and is required for female fertility in the mouse [1]. mRNA expression pattern of TAF4b is more restricted than its paralog, TAF4, which is highly expressed in many tissues. Low levels of TAF4b mRNA can be detected in most tissues, but it is enriched in the spleen, ovary and testis. *In situ* hybridization has revealed that within the mouse ovary, TAF4b is highly expressed in proliferating granulosa cells, while TAF4 has a more uniform somatic distribution and a strong signal in oocytes [1]. Why both of these TFIID components are present and differentially expressed in the ovary, and what specific roles each may be performing there, is unknown.

While TAF4 is essential for embryogenesis, mice with a targeted deletion of TAF4b are viable with developmental defects only observed within the gonad [1-3]. TAF4b-null males are initially fertile, but become sterile by 3 months of age, arising from an inability to maintain spermatogonial stem cells [3]. The etiology of TAF4b-null female infertility is not as straightforward, displaying pleiotropic defects including elevated FSH levels at postnatal day 23 and a reduction of primordial germ cells at postnatal day 3, indicating deficiencies exist before the onset of puberty [4]. In the

neonatal mouse, TAF4b protein has been detected in germ cells but not the somatic cells of primordial follicles [4]. In the adult ovary, we have reported high expression of TAF4b mRNA in granulosa cells [1, 5]. It is likely that TAF4b is expressed in both cell types but may be temporally or otherwise developmentally restricted.

A specific block in ovarian follicle development is not observed in TAF4b-null females, but rather, a decrease in follicle number at all developmental stages, with the exception of an increased number of atretic follicles [5]. The majority of follicles recruited into development will not be ovulated, but will instead undergo an apoptotic death termed 'atresia,' while only a very few dominant follicles are selected for complete maturation. Apoptosis in granulosa cells can be induced through death ligand-receptor signaling or by the lack of growth factors or cytokines. It is likely that the gonadotropin signals that promote proliferation in the dominant follicles also induce apoptosis in subordinate follicles. Cells that receive proliferation signals without proper expression of survival factors do not proliferate, but die by apoptosis. While atresia is a normal part of ovarian function in fertile mammals, elevated atresia in TAF4b-null ovaries could indicate that there is a problem in granulosa cell survival.

Key ovarian survival factors include Akt and the Foxo subfamily of forkhead transcription factors, Foxo1, Foxo3a and Foxo4. Upon signaling through phosphatidylinositol 3-kinase (PI3-K), Akt phosphorylates multiple targets, including Foxo proteins, to promote granulosa cell survival [6]. In the absence of Akt activation, Foxo proteins translocate to the nucleus and increase gene expression of pro-apoptotic genes such as Fas-L and Bim [7, 8]. The PI3-K pathway is negatively regulated by phosphatase and tensin homolog (Pten). Granulosa cell-specific deletion of Pten in mouse

results in reduced apoptosis, increased proliferation and survival, due in part to reduced repression by Foxo1 [9]. Together, the Akt, Foxo and Pten proteins coordinate to determine the rate of recruitment and fate of growing follicles.

The same signals that are required for granulosa cell survival are required in part for FSH-mediated proliferation. Upon signaling from FSH and the TGF-β family member activin intracellular cAMP levels are elevated and the PI3-K pathway is activated, which is necessary to remove Foxo1 repression of the cyclin D2 promoter [10]. Expression of cyclin D2 promotes the G1 to S transition in granulosa cells and is required for the burst of proliferation between secondary and antral follicle stages [11]. The initial gene characterization of expression defects in adult TAF4b-null females indicated a down-regulation of cyclin D2 [1]. In addition to FSH, the steroid hormone, estrogen, promotes cell proliferation and can also exert an anti-apoptotic effect over follicles. In cattle, exogenous administration of estrogen has been observed to decrease Fas-L mediated apoptosis, increase cyclin D2 expression, and increase BrdU incorporation into granulosa cells [6]. An improper balance of the factors that respond to proliferative signals could result in the diverse fertility defects observed in TAF4b-null females.

In addition to their individual roles, granulosa cells and oocytes must be able to communicate with each other for the generation of a healthy oocyte. The oocyte and surrounding granulosa cells are connected by gap junctions composed of connexin 37 (Cx37), and these channels are necessary for both oocyte-mediated regulation of granulosa cells, and transfer of small molecules from granulosa cells to the oocyte [12, 13]. The layer of granulosa cells directly surrounding the oocyte are known as cumulus granulosa cells, while the ones closer to the basal lamina are called mural granulosa cells.

Oocyte secreted factors maintain the phenotypic differences between cumulus and mural granulosa cells which include the higher proliferation rate, lower sterodiogeneic capacity and very low LHR expression in cumulus cells. Oocytes produce and secrete paracrine signals such as GDF9 and BMP15 to cumulus cells preventing their premature luteinization and creating a microenvironment that allows for the later states of oocyte maturation [14-16]. Communication between granulosa cells within the follicle is also a requirement of successful folliculogenesis, and is mediated by connexin 43 (Cx43) containing gap junctions [17, 18]. Deletion of Cx43 from the mouse ovary prevents the expansion of the granulosa cell population at early stages in folliculogenesis, preventing oocyte maturation [19]. Because TAF4b-null ovaries express many of the important granulosa cell and oocyte genes tested *in vivo*, we question if the communication system between these cells is intact.

A number of TAF4b-target genes have been identified or verified using cell culture systems involving over-expression of FLAG-tagged TAF4b. Among these are several granulosa-cell specific transcripts such as inhibin α, inhibin βB, inhibin βA, cyclin D2, follistatin as well as the universally expressed activator, c-Jun [20]. Transfection of FLAG-tagged TAF4b into NIH3T3 cells, which are not of granulosa cell origin, induces the expression of granulosa cell-specific genes, suggesting that expression of TAF4b is sufficient to induce granulosa cell-like gene expression. In addition, transfection of FLAG-tagged TAF4b into SIGC (rat granulosa cell line), but not 3T3 cells, induces c-Jun expression, introducing a novel, cell-type specific ability of TAF4b to control a ubiquitously expressed gene. ChIP experiments have verified the localization of TAF4b to the promoters of c-Jun, cyclin D2, follistatin and inhibin βA. TAF4 is also

present at c-Jun and cyclin D2 promoters. While there is strong evidence that these genes are direct TAF4b targets, it remains unknown how they are regulated in the TAF4b-null ovary, and if their misregulation is an underlying cause of the infertile phenotype.

Given TAF4b's mRNA expression pattern, we hypothesize that loss of TAF4b affects granulosa cell function, contributing to infertility. To examine this possibility, we have quantified levels of apoptosis, hormone-induced proliferation, survival gene expression and connexin localization in the TAF4b-null mouse and its fertile littermates. We also verified expression of TAF4b target genes in juvenile ovaries and how siRNA-mediated knockdown of TAF4b and TAF4 affects expression in cultured granulosa cells. Although granulosa cell survival and proliferation is compromised in the TAF4b-null ovary, data presented here indicate granulosa cell defects are not likely the sole cause of infertility.

Results

TAF4b protein is enriched in ovarian granulosa cells

RNase protection assays had previously demonstrated elevated mRNA expression levels of TAF4b in the testis, and to a lesser degree, the ovary [1]. To extend TAF4b tissue-enriched expression to the protein level, we performed Western blot analysis on adult mouse tissue samples (Figure 1A). The overall TAF4b protein expression patterns are highly similar to their mRNA profiles. TAF4b protein was highest in the testis, closely followed by ovary and spleen, with low levels of TAF4b protein observed in the majority of other tissues tested. We then tested if TAF4b protein is enriched in ovarian granulosa cells, as was previously determined for its mRNA expression by *in situ* hybridization [1].

Whole ovaries from 8 week-old wildtype mice were subject to needle puncture to release granulosa cells. Protein extracts were made from the isolated granulosa cells, the ovarian remnants following granulosa cell collection, and total ovary and used in Western blot analysis. We found TAF4b enriched in purified granulosa cells of the adult ovary and, conversely, depleted in ovarian remnants after granulosa cell retrieval, in agreement with the previously reported *in situ* mRNA expression pattern (Figure 1B).

TAF4b-null ovaries exhibit increased apoptosis

Granulosa cell survival is critical for oogenesis. Although a background level of atresia is observed in healthy ovaries, TAF4b-null ovaries contain more atretic follicles than wildtype littermates [5]. To directly analyze follicular atresia on a molecular level we assessed caspase-3 activation in granulosa cells, which is a reliable marker of apoptosis in this cell type [21]. We immunostained ovary sections from young, 3–4-week-old TAF4b-null and wildtype or TAF4b heterozygotes with procaspase 3 and cleaved-caspase 3 antibodies. Procaspase 3 staining was detected uniformly throughout the ovaries, and appeared diffuse in the cytoplasm, and granular in the nucleus, as previously described [22] (Figure 2A). In atretic, but not healthy follicles, cleaved-caspase 3 can be detected in a subset of cells (Figure 2 B). This assay detected increased granulosa cell apoptosis in the pre-antral follicles of TAF4b-null ovaries as compared to wildtype or heterozygous controls. Quantification of positively stained cells in the pre-antral follicles confirmed a significant increase of apoptotic cells in TAF4b-null ovaries (Figure 2C).

Impaired granulosa cell proliferation after hormonal stimulation in TAF4b-null mice

TAF4b-null ovaries contain more atretic follicles than their wildtype or heterozygous littermates, while their number of pre-antral and antral follicles is reduced [5]. To better understand the cellular basis of the observed folliculogenesis defects in the TAF4b-deficient ovaries, we assessed the proliferation of granulosa cells in TAF4b-null and heterozygous ovaries. Bromodeoxyuridine (BrdU) incorporation *in vivo* was used to directly label granulosa cells in the S-phase of the cell cycle as a measure of granulosa cell proliferation. Twenty-four hours prior to BrdU labeling, matched 21 day old TAF4b-null and heterozygous female mice were stimulated with a single injection of either estrogen [23] or PMSG [24]to induce proliferation of follicular granulosa cells (Figure 3A-D). Ovaries were harvested the next day following 1 hour *in vivo* BrdU treatment, and ovary sections generated from these mice were labeled with anti-BrdU antibodies. BrdU incorporation was readily detectable in granulosa cells derived from heterozygous mice and significantly reduced, but not absent, in the TAF4b-null ovaries in response to both hormonal treatments (Figure 3E).

TAF4b-null follicles have a reduced capacity to proliferate upon exogenous hormonal stimulation. To ensure that this effect was not due to transcriptional misregulation of the hormone receptors, we assayed levels of FSH receptor (Fshr) and estrogen receptor β (Esr2) in TAF4b-null, wildtype and heterozygous ovaries by qRT-PCR. To mimic conditions for the BrdU incorporation assay, we tested estrogen-primed 3 week-old TAF4b-null and heterozygous ovaries and found no reduction of either transcript (Figure 4). The TAF4b-null follicles are unable to respond to FSH and

estrogen at a point downstream of receptor mRNA expression and, likely, downstream of hormone-receptor binding.

Normal connexin localization in TAF4b-null mice

Bi-directional communication is crucial to granulosa cell and oocyte function and survival. To assess the TAF4b-null ovary's capacity for inter-cellular communication, we used immunofluorescence to localize connexin proteins in the ovaries of 4 week-old TAF4b-null and heterozygous mice (Figure 5). Both TAF4b-null and heterozygous ovaries show proper localization of connexins 37 and 43, suggesting that the channels responsible for inter-cellular communication are intact.

Immunostaining with connexin 43 allowed us a new view of the follicular structures in TAF4b-null ovaries by highlighting all of the follicles within an ovarian section. We noticed that the 4 week-old TAF4b-null ovary contained large, antral follicles. Previous studies had not observed many large, multi-layered and healthy follicles in TAF4b-null ovaries and had attributed that phenotype to an inability of the knockout to undergo FSH-induced proliferation. Although the numbers of such follicles are reduced in the TAF4b-null ovary, the fact that they are able to form in the absence of TAF4b led us to question the replicative capacity of TAF4b-null granulosa cells upon hormonal stimulation. One point of confusion with this observation was that during the initial characterization of the TAF4b-null ovaries, cyclin D2 levels were found to be dramatically reduced. Cyclin D2 is an essential factor for female fertility and is required for the granulosa cell proliferation events that lead to antral follicle formation. To test if cyclin D2 is actually present in the ovaries of 4 week-old TAF4b-null granulosa cells, we used qRT-PCR and Western blot analysis (Figure 6). There is a slight reduction of cyclin

D2 mRNA in the TAF4b-null granulosa cells, but it is not statistically significant. mRNA levels of p27, the CDK inhibitor that prevents cyclin D2-induced proliferation [25], is unchanged between TAF4b-null and wildtype granulosa cells. TAF4 mRNA levels are also similar between TAF4b-null and wildtype granulosa cells, suggesting that an upregulation of TAF4 is not compensating for loss of TAF4b, although this finding would have to be extended to the protein level to make a definitive statement. Cyclin D2 protein is present at equivalent levels in the ovaries of TAF4b-null and wildtype mice, ruling out loss of cyclin D2 at 4 weeks of age as an underlying cause of TAF4b-null infertility.

Target gene reduction following TAF4b knockdown in NT-1 cells

Cyclin D2 has been identified as one of many TAF4b-target genes using FLAG-tagged TAF4b over-expression and ChIP assays [20]. While it does not appear that TAF4b is required for cyclin D2 expression *in vivo*, we wanted to examine TAF4b's influence on cyclin D2 and other target genes in a pure population of granulosa cells. We used siRNA-mediated knockdown of TAF4b, as well as TAF4, in the NT-1 mouse granulosa cell line and assayed target gene expression. NT-1 cells were transfected with siRNAs against TAF4, TAF4b and a non-targeting siRNA, and harvested at 60 hours. TAF4 and TAF4b protein levels were specifically reduced while the non-targeting siRNA did not affect TAF levels (Figure 7A). Knockdown of TAF4b reduces the expression of c-Jun, cyclin D2, inhibin βA and Igfbp-3, which agrees with previous data (Figure 7B). Of the four target genes tested, cyclin D2's reduction upon TAF4b knockdown was the most modest with about a 25% reduction. Inhibin βA was decreased 45%, c-Jun, 40%, and Igfbp-3, 55%. Interestingly, reduction of TAF4 also reduced the target genes with the

exception of inhibin βA. Upon TAF4 knockdown cyclin D2 was reduced 40%, c-Jun, 30%, and Igfbp-3, 40%. These data confirm TAF4b targets, but also introduce the idea that TAF4 and TAF4b may work together to promote the correct expression of these granulosa cell transcripts.

Age-dependent reduction of survival factors in TAF4b-null ovaries

The initial characterization of TAF4b-null gene expression patterns was based on comparison of adult wildtype and knockout ovaries. We now know that adult TAF4b-null ovaries have far fewer granulosa cells compared to younger knockouts and gene expression changes between the two ages reflect the loss of granulosa cells with age. To determine if there were disruptions that may underlie TAF4b granulosa cell depletion we assayed the levels of several Foxo/Akt/Pten pathway genes from TAF4b-null and wildtype ovaries at 4 and 16 weeks of age (Figure 8 A). To detect the levels of these proteins, we used antibodies against Foxo1, total Foxo3a, Foxo3a phosphorylated at Ser 318/321, at Thr32, at Ser253 and total Foxo4 in Western blots of whole mouse ovary protein extracts. At 4 weeks of age, all the Foxo proteins tested are present at near equivalent levels between the wildtype and TAF4b-null ovary. In 16 week-old TAF4b-null ovaries, Foxo1 and phosphorylated Foxo3a are nearly undetectable, while total Foxo3a and Foxo4 are present but with reduced levels. Immunofluorescent detection of Foxo1 reveals that at 4 weeks of age TAF4b-null ovaries contain multiple follicles expressing Foxo1, but by 16 weeks, TAF4b-null ovaries are depleted of such follicles (Figures 8B and 8C). Although there is almost complete loss of Foxo3a phosphorylation in the 16 week TAF4b-null ovary, there is only about a 50% loss of total Foxo3a protein, suggesting that at 16 weeks, there is reduced Foxo3a phosphorylation in TAF4b-null

ovaries. To examine upstream factors, we used antibodies against Pten, multi-phosphorylated Pten, total Akt, and Akt phosphorylated at Thr308 and at Ser473 (Figure 8). There is a slight decrease in both total and phosphorylated Akt at 16 weeks in the TAF4b null ovary. Pten and p-Pten are equivalent between genotypes at both 4 and 16 weeks. p27 levels are equivalent at 4 weeks and elevated in the 16 weeks old knockout. At the earliest time point examined, defects in the Akt survival pathway were not prevalent and these later defects may be a consequence of, rather than the cause of, follicle depletion of the TAF4b-null ovary.

Granulosa cell-derived defects are not the only cause of follicle death. Oocyte factors play critical roles in maintaining the health of the follicle. Preliminary *in situ* data from adult ovary indicated that TAF4b was transcribed in granulosa cells, but from that experiment it was not clear if TAF4b was also transcribed in oocytes. To determine if TAF4b is present in adult oocytes, we used qRT-PCR to compare the level of TAF4b and TAF4 using cDNA from oocytes and tissues with high TAF4b expression (Figure 9). TAF4b appears to have equivalent expression between oocytes and total ovary. This is a relatively high expression with a value 3-fold over spleen, and only 30% less than in the testis. TAF4 expression shows a different profile, with the highest expression in the oocyte- nearly two-fold higher than that of total ovary.

Discussion

Conflicting reports have localized TAF4b exclusively to the granulosa cell and ooctye [1, 4]. The initial *in situ* performed for both TAF4 and TAF4b in the adult ovary demonstrated that while TAF4 was more ubiquitously expressed and displayed high

expression in the oocyte, TAF4b expression appeared restricted to granulosa cells [1]. Although *in situ* hybridization is a very informative tool, it is not a quantitative assay, and the presence of TAF4b in the oocyte was never directly tested. Here we demonstrate using qRT-PCR that TAF4b transcript is present in the oocytes of growing follicles. Although it would be useful to know TAF4b protein expression at different follicular stages, we are limited by the TAF4b antibody available to us, which does not work very well in immunofluoresence (data not shown). Use of another TAF4b antibody in a different study demonstrated that the protein was present in primordial ovarian follicles, but not granulosa cells in neonatal mice [4]. It is likely that TAF4b is not transcribed in the pre-granulosa cells of primordial follicles, but that its expression is induced after the follicle is recruited into development. Granulosa cells isolated from needle puncture, as performed here, are derived from large, growing follicles, and we have shown by Western blot that TAF4b protein is abundant in this purified population. Together, these data suggest that TAF4b is expressed in both oocytes and granulosa cells in a dynamic manner that is dependent on follicular stage.

Individually, granulosa cell and oocyte functions are crucial for folliculogenesis and oogenesis, but coordination between the two cell types is equally important. Targeted deletion of either gap junction protein, Cx37 or Cx43 (ovary-specific), results in female infertility [12, 19]. TAF4b-null ovaries are able to transcribe the vast majority of important granulosa cell and oocyte genes yet still have impaired folliculogenesis and increased atresia leading us to question if the proper gap junctions were in place. Cx37, which is localized between the oocyte and its surrounding cumulus granulosa cells is required for oocyte maturation at the late antral stage of folliculogenesis. Cx37 knockout

mice rarely display antral follicles, do not ovulate and undergo premature luteinization. TAF4b-null mice have impaired ovulation but are still capable of forming corpora lutea. However, at 4 weeks of age it appears that Cx37 gap junctions are forming correctly in the TAF4b-null females. Cx43 is required for inter-granulosa cell communication and proliferation and is particularly important for the transfer of signaling molecules following hormonal stimulation [26]. When we observed a defective response to exogenous hormone administration, we hypothesized that granulosa cell communication may have been compromised, however, Cx43 gap junction formation in TAF4b-null granulosa cells appears normal. The relationship between Cx43 and apoptosis is complex in that it has been found to both promote and prevent death in a variety of cell types [27-30]. It seems that Cx43 allows for the transfer of both survival and death signals throughout coupled cells, such as in the ovarian follicle, to promote or prevent apoptosis in the entire unit. Although an attractive prospect given the relatively normal expression of so many granulosa cell and oocyte genes, defects in connexin channels do not appear to be underlying infertility in TAF4b-null females.

What was apparent when comparing ovary sections of 4 week-old TAF4b-null and heterozygous mice, was that TAF4b-deficient ovaries, at some low level, are able to form large and healthy looking follicles. While previous reports have demonstrated that TAF4b-null oocytes are not able to undergo *in vitro* cumulus expansion, and we show here that upon administration of exogenous hormones TAF4b-null follicles do not proliferate well, somehow some antral follicles can form under natural conditions. The elevated FSH level in TAF4b-null mice could be responsible for this disparity. While fertile mice only have transient peaks of FSH during the estrous cycle, TAF4b-null mice

have levels of FSH that are much higher than even peak levels of wildtype mice. High FSH levels are also observed in infertile women, and increased FSH levels are inversely correlated with successful IVF treatment. To overcome this limitation, clinicians have attempted to increase doses of FSH to induce ovulation in women, but have not been successful [31]. In fact, ovulation regimens in mice have uncovered that too much PMSG reduces the number of oocytes released and that correspondingly high doses of LH are necessary for ovulation. During the menopausal transition women display elevated FSH levels but still have occasional, irregular ovarian cycles where eggs mature but may not be ovulated [32]. High FSH levels, inability to induce follicle maturation with administration of exogenous hormones, and anovulation are observed in the TAF4b-null female, indicating that young TAF4b-null female mice display many of the same characteristics as a peri-menopausal woman.

Another characteristic of fertility defects that arises in women as they approach menopause is poor oocyte quality. Atresia is often thought of as an oocyte quality selection mechanism. Many more follicles are recruited into development than are ovulated, and it is evolutionarily advantageous to ensure that the healthiest oocyte is available for fertilization. Granulosa cell apoptosis usually initiates atresia in the normal selection pathway, but some conditional knockout studies in mouse oocytes also lead to follicle death. TAF4b-null mice experience above-normal levels of atresia, as demonstrated by a significant elevation of cleaved-caspase 3, and it is unknown if the defect leading to increased atresia is derived from oocyte or granulosa cell defects, or a combination of both.

The initial microarray comparing adult TAF4b-null to wildtype ovaries revealed a down-regulation of a number of granulosa cell specific genes. Follow-up studies have shown that over-expression of TAF4b in the non-granulosa cell line, NIH3T3, is sufficient to induce transcription of some granulosa cell specific transcripts such as inhibin α, inhibin βB, inhibin βA and cyclin D2. ChIP data has even localized TAF4b to the promoters of inhibin α and cyclin D2 but a subsequent study shows that these genes are still transcribed properly in the young TAF4b-null ovary. Here we show that selective siRNA mediated knockdown of TAF4b is able to reduce transcription of c-Jun, cyclin D2, inhibin βA and Igfbp-3. Interestingly, knockdown of TAF4 reduces the level of c-Jun, Igfbp-3, and cyclin D2 as well. TAF4 and TAF4b have not been shown to be mutually exclusive of each other within TFIID, but it is known that only one of these proteins is required for the complex to assemble [2]. It is possible that TAF4 and TAF4b are present in the same complex to activate gene expression. Indeed, the same experiment that localized TAF4b to the cyclin D2 promoter also determined that TAF4 also occupied the promoter, as opposed to inhibin βA, which exhibited a weak signal for TAF4 and a strong signal for TAF4b [20]. The data presented here agree with the ChIP data in that knockdown of TAF4 greatly reduces the expression of cyclin D2 while it does not affect inhibin βA expression, and TAF4b knockdown reduces the levels of both cyclin D2 and inhibin βA. Although TAF4b reduction in NT-1 cells reduces cyclin D2 transcript, cyclin D2 protein is present in 4 week-old TAF4b-null ovaries. Cyclin D2 is a critical granulosa cell proliferation factor, and the level of folliculogenesis achieved in the 4 week-old TAF4b-null ovary would not be possible without appreciable levels of cyclin

D2, suggesting that there are TAF4b-independent mechanisms to ensure expression of cyclin D2 in granulosa cells *in vivo*.

In an attempt to find early underlying factors that may be disrupted leading to proliferative defects or granulosa cell death in the adult TAF4b-null ovary, we examined the Pten/Akt/Foxo pathway. Disruption of the components of this pathway in the TAF4b-null ovary could explain the increase in apoptosis and reduced proliferation in response to FSH [5]. If this pathway were mis-regulated at an early time-point in development, it may explain the loss of follicles in the mature TAF4b-null ovaries. However, at 4 weeks, Pten, Akt, and Foxo proteins are present and appropriately phosphorylated in TAF4b-null ovaries at levels very similar to wildtype signifying that this pathway is largely intact. As expected, Foxo1 is nearly undetectable in the 16-week TAF4b-null ovary given that Foxo1 expression is concentrated in the granulosa cells of growing follicles [33] and by this time-point the TAF4b-null ovary is largely devoid of such follicles. Foxo3a is reduced in the 16 week-old TAF4b-null ovary, but unlike the complete loss observed with Foxo1, Foxo3a is present at about 50% of the wildtype level. There appears to be a near total reduction in Foxo3a phosphorylation at 16 weeks, which supports our observation of increased apoptosis. The cell cycle inhibitor, p27, appears slightly increased in the 16 week-old knockout, which agrees with loss of growing granulosa cells and an ovarian composition of mostly non-dividing stromal and differentiated granulosa cells in the older TAF4b-null ovary. Overall, the protein expression changes in TAF4b-null ovaries indicate a loss of survival factors by 16 weeks, but the disruptions in this pathway appear to follow, and not precede, ovary deterioration in the TAF4b knockout.

The data presented here introduce a new aspect of the TAF4b-null phenotype, which is a surprisingly normal ovary, both in appearance and gene expression, at 4 weeks of age. Although there are several important granulosa cell genes that TAF4b regulates, it does not appear to be required for their expression in the ovary of the young TAF4b-null mouse. Although we note a drastic reduction in survival factors in the TAF4b-null ovary from 4 to 16 weeks, we do not know the underlying cause of ovarian degeneration. We demonstrate here that while many aspects of the young TAF4b-null ovary appear normal, by 8 weeks, when the mice are old enough to breed, there are signs of ovarian degeneration. Compared to fertile littermates, TAF4b-null mice experience increased levels of granulosa cell apoptosis, decreased granulosa cell proliferation upon administration of exogenous hormones and decreased Foxo3a phosphorylation.

Together the data suggest that we are still missing an aspect of TAF4b-dependent regulation that leads to absolute female infertility. Verification of TAF4b transcript in the oocyte suggests that infertility could stem from either cellular compartment, or be a combination of deficient TAF4b-dependent gene expression from both cell types. Closer examination of the gene expression profile in juvenile TAF4b-null ovaries could provide very useful information into the underlying infertility seen here, and perhaps lead to a better understanding of mammalian fertility pathways.

Materials and Methods

Purified granulosa cell isolation

The ovaries of 8 week old wild type C57/Bl6 mice (Jackson Laboratories, Bar Harbor, ME) were removed and subjected to puncture with a 26 gauge needle to liberate granulosa cells into collection media (DMEM-F12, 1X penicillin-streptomycin, 0.3%

BSA). Resulting granulosa cell suspensions were filtered through 40 μm nylon filter to remove large tissue clumps and oocytes. The granulosa cell-depleted residual ovarian tissue (termed GCR here) was washed in PBS and homogenized in a Dounce homogenizer in low salt protein extraction buffer (0.2M KCl, 0.1M Tris, pH 8, 0.2mM EDTA, 0.1% NP-40, 10% glycerol, 1mM PMSF). Purified granulosa cells were spun down, washed in PBS, spun down again, and re-suspended in extraction buffer for subsequent Western blot analysis, or processed using the micro Rneasy kit (Qiagen) for RNA isolation.

Western Blot analysis

Tissues were Dounce homogenized and soluble protein extracted in low salt buffer. Protein concentration was determined by Bradford assay and equivalent amounts of protein were loaded per lane. The following antibodies were used: Cell Signaling Technologies, (Danvers, MA) Foxo1 #2880, Phospho-Foxo1 (Thr24)/Foxo3a (Thr32) #9464, Foxo3a #9467, Phospho-Foxo3a (Ser253) #9466, Phospho-Foxo3a (Ser318/321) #9465, Foxo4 #9472, Akt #9272, Phospho-Akt (Ser473) #9271, Phospho-Akt (Thr308) #9275, Pten #9552, Phospho-Pten (Ser380/Thr382/383) #9554, BD Transduction Laboratories (San Jose, CA), TAF4 (TAF135) #612054, Novus Biologicals (Littleton, CO) β-Tubulin RB-9249-P, Santa Cruz Biotechnology (Santa Cruz, CA) p27 (C-19) sc-528, Labvision (Fremont, CA) Cyclin D2 #MS-214, and TAF4b[5].

BrdU incorporation

Assessment of granulosa cell proliferation by BrdU incorporation was performed as previously described by [23] . Hormonally primed mice (5 IU PMSG or 1 μg/g E2 for 24 hours) were injected ip with 1 μg/gm BrdU, and sacrificed after 1 hour. The ovaries were

dissected, frozen in OCT, and 7 μm frozen sections were generated. The sections were rinsed in CSK buffer (10 mM Hepes pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$) once at room temperature, incubated in CSK/Tx-100 (0.5%) buffer for 2 minutes on ice, and fixed in 100% methanol at -20°C for 20 min. The DNA was denatured by incubation in 2N HCl for 90 min at room temperature, and the slides were neutralized by rinsing 2x w/PBS. Endogenous peroxidase activity was quenched by incubating in 3% H_2O_2 for 5 min. The slides were further washed in PBS, and blocked in PBS/0.5% Tween-20 with 10% normal goat serum and 10 mg/ml BSA for 20 min followed by blocking with PBST supplemented with 200 ug/ml unlabeled goat anti-mouse Fab fragments. Primary monoclonal anti-BrdU antibody (Sigma) was used at 1:100 dilution; the remaining procedures were carried out as described above. After counterstaining with hematoxylin, the percentages of positive cells per follicle were determined for comparison in 5 follicles per each experimental animal. Preantral follicles were analyzed in the estrogen-treated animals; in the PMSG-treated group, antral follicles induced by hormonal treatment in wildtype and heterozygous animals were included into the analysis as well.

Oocyte isolation

Ovaries from 8 week-old wildtype mice were removed, cleaned of excess fat and bursal sac and placed in enzyme solution (0.1% collagenase type 3 (Worthington), 0.02% Dnase type I (Worthington) and 0.1% trypsin (Invitrogen) in Hanks Balanced Salt Solution (Invitrogen)) and agitated for 30 minutes at 37°. Ovaries were dispersed using a 20G needle, the solution was diluted with DMEM-F12 and oocytes were collected. Oocytes

were washed once with PBS and resuspended in Trizol reagent for subsequent RNA isolation. Ovaries of 3 mice, approximately 120 oocytes, were combined per sample.

Tissue isolation

Ovaries, testis and/or spleens were removed from 8 week-old wildtype mice, rinsed in PBS and dounced in Trizol reagent for subsequent RNA isolation, or in extraction buffer for protein isolation.

Quantitative RT-PCR

Total RNA was isolated from indicated tissues using Micro RNeasy kits (Qiagen) or Trizol reagent (Bio-rad) and concentration was determined using a Nanodrop. 20 μl of cDNA was prepared from approximately 500 ng of total RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's directions, using a mix of oligo(dT) and random primers. Real-time PCR reactions were performed in triplicate using 2 μl cDNA template, ABI Power SYBR green PCR master-mix and gene specific primers in the ABI 7500 Real Time PCR System. Dissociation curves were generated and PCR products were run on an agarose gel to verify amplification of a single product. qPCR data were analyzed by the ΔΔCt method. Relative expression levels were normalized to L-17 mRNA or 18S ribosomal RNA levels to correct for equivalent total RNA levels as indicated.

Quantitative RT-PCR primers

	Forward (5'-3')	Reverse (5'-3')
TAF4b	GAT GTT ACT AAA GGC AGC CAA GAG T	CTG CTC TGG ATC TTC TTT ATT GGA G
18S rRNA	CCG CGG TTC TAT TTT GTT GG	GGC GCT CCC TCT TAA TCA TG
TAF4	ATC TCC ACT GTG CAG GCTT CC	GGT CAG CTG CCG TGC AAT A
Cyclin d2	GCT CTG TGC GCT ACC GAC TT	AAT CAT CGA CGG CGG GT
c-Jun	CCC CTG TCC CCT ATC GAC AT	TTT TGC GCT TTC AAG GTT TTC
Igfbp-3	CGG GAG ACA GAA TAC GGT CC	GCT TTC TGC CTT TGG AAG GG
Inhba	TGA ACT CAT GGA GCA GAC CT	GAC AGG TCA CTG CCT TCC TT
p27	GTC CAG GGA TGA GGA AGC G	CCT TTT GTT TTG CGA AGA AGA ATC
L-17	GCA AAC CAG AAC TAA GGA AAA AGG T	CGT TGT CGA ATT ACC ACT GCT G
Esr2	AAA CAG AGA GAC CCT GAA GAG AA	CCT CTT GGC GCT TGG ACT AG
Fshr	CCA ACC TAC CCA ACT TGC ATG	CAG ATA TCG GAG ACT GGG AAG ATT

Immunofluoresence

Caspase-3 detection: Frozen ovarian sections of estrogen-primed TAF4b-null, heterozygous and wildtype mice fixed in methanol were labeled with rabbit monoclonal antibodies against cleaved caspase-3 (5A1) and against total caspase-3 (8G10) (Cell Signaling Technology, Danvers, MA).

Connexin detection: Frozen ovarian sections from 4 week-old mice were fixed in 4% paraformaldehyde. Primary antibodies used were connexin 37 (Zymed) and connexin 43 (Sigma). Alexa-fluor 594 conjugated secondary antibody was used, followed by mounting in DAPI mounting medium (Vectasheild). Images were obtained on the Zeiss Imager M1 and processed with Axiovision.

Foxo1 detection: 4 and 16 week-old TAF4b-null ovaries were treated as described for connexin staining. Foxo1 #2880 Cell Signaling Technologies, (Danvers, MA) primary antibody was used.

siRNA-mediated gene knockdown

TAF4 and TAF4b SMARTpool siRNAs and ON-TARGET*plus* Non-Targeting Pool (mouse) were purchased from Dharmacon and transfected into the mouse NT-1 cell line using Dharmafect reagent 1. Optimization was performed per manufacturer's instructions. Cells were harvested at 60 hours post-transfection and prepared for Western blot anlaysis or qRT-PCR.

Acknowledgements

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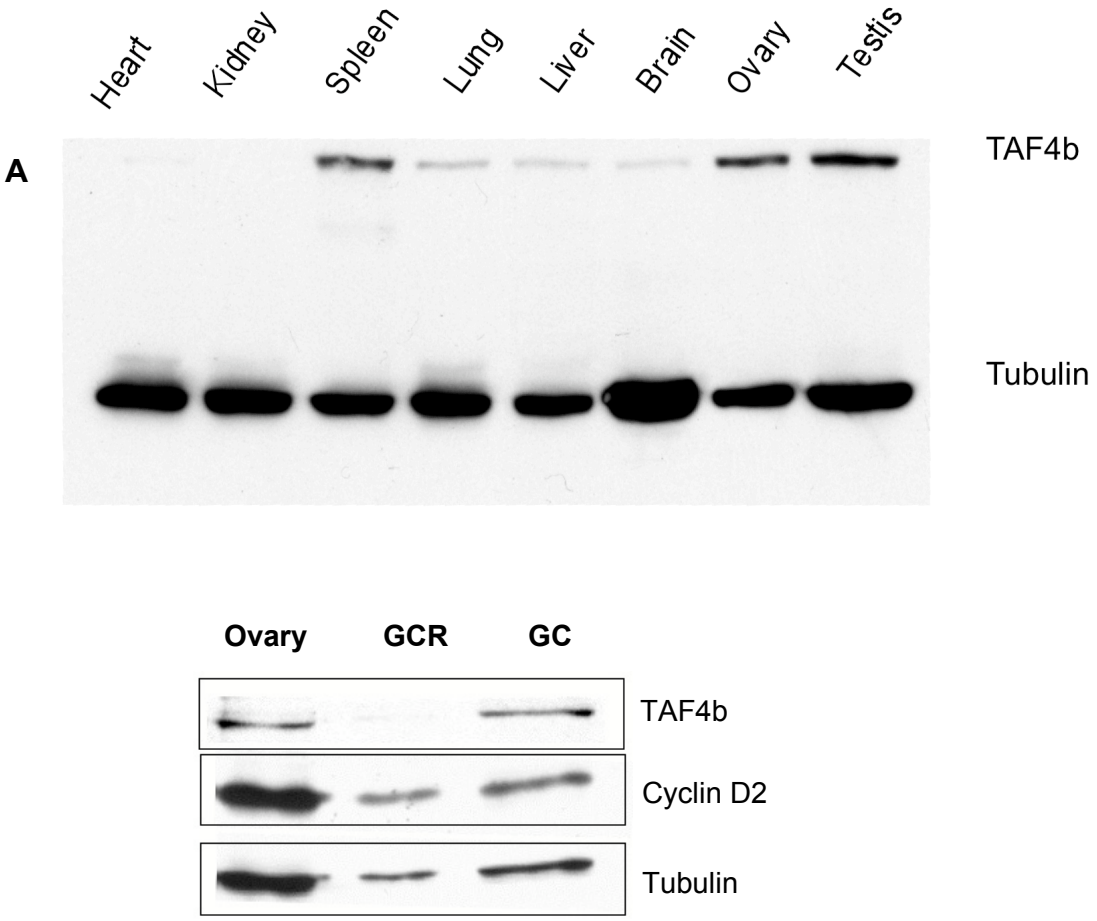
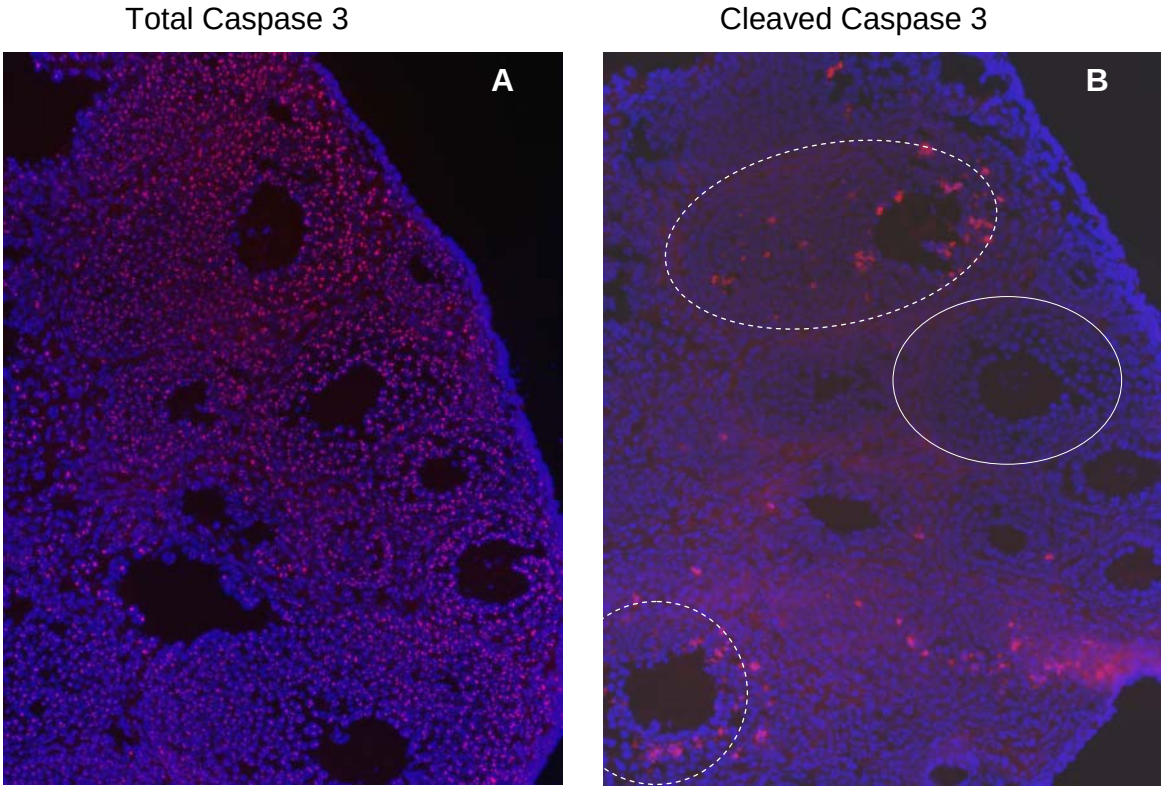


Figure 1. TAF4b protein is enriched in ovarian granulosa cells. (A) Western blot of mouse tissue extracts probed with anti-TAF4b antibodies. (B) TAF4b is enriched in purified granulosa cells (GC), as opposed to ovary remnants depleted of granulosa cells (GCR). Total ovary protein extract represents starting material without fractionation. Cyclin D2 is a granulosa cell-enriched cell cycle protein. Tubulin serves as a loading control.



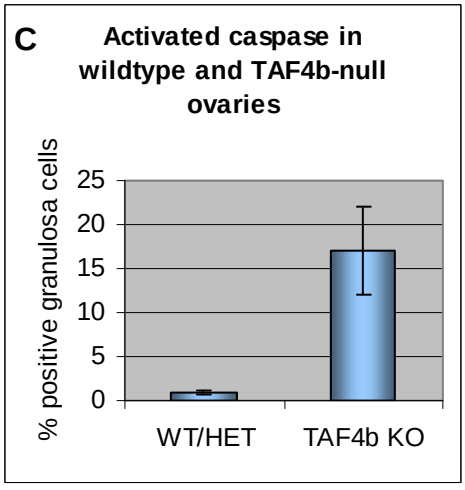


Figure 2. Increased apoptosis in TAF4b-null ovaries. Immunofluorescent detection of (A) total caspase 3 and (B) cleaved caspase 3 in TAF4b-null ovary. Atretic (dashed line) and non-atretic (solid line) growing follicles are circled. Red= caspase, Blue= DAPI

(C) Quantification of percent positively stained granulosa cells from growing follicles of wildtype (WT) and TAF4b heterozygotes (HET) compared to TAF4b knockouts (KO).

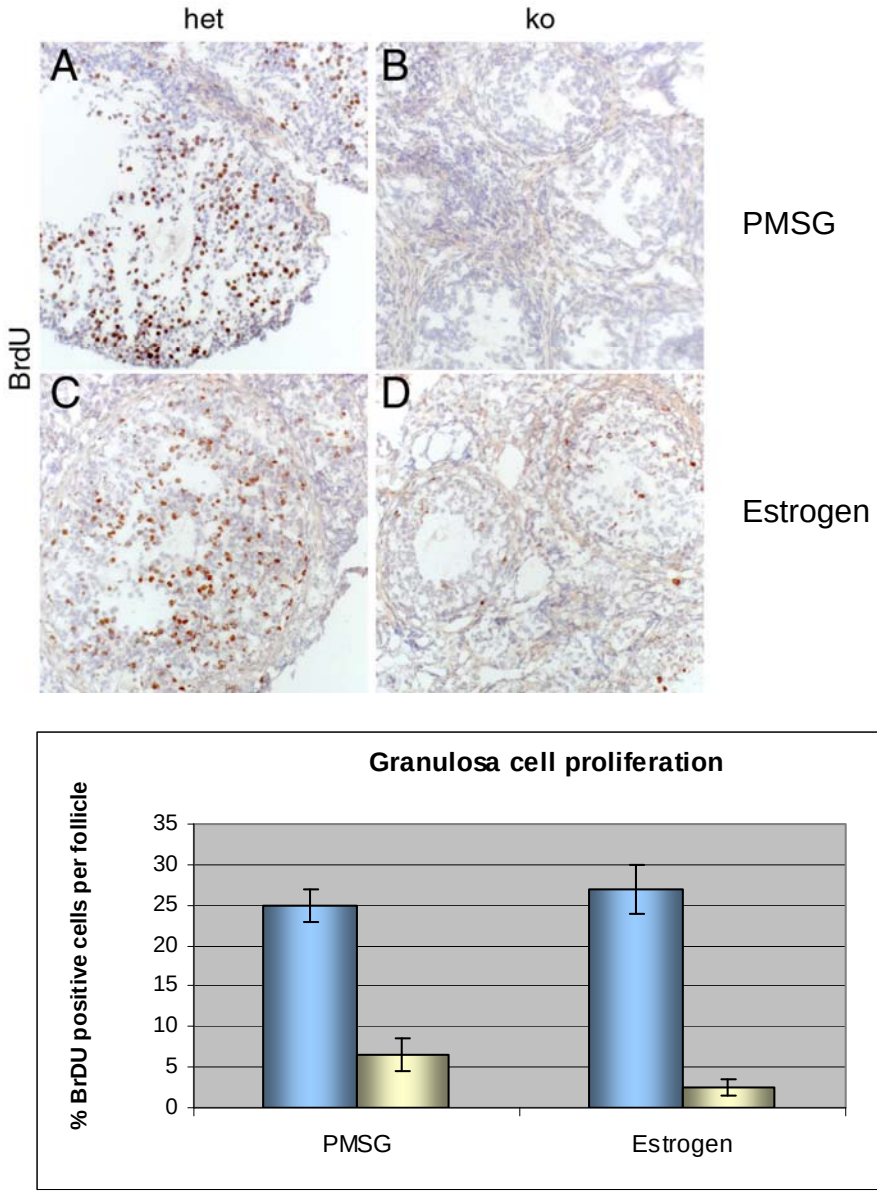
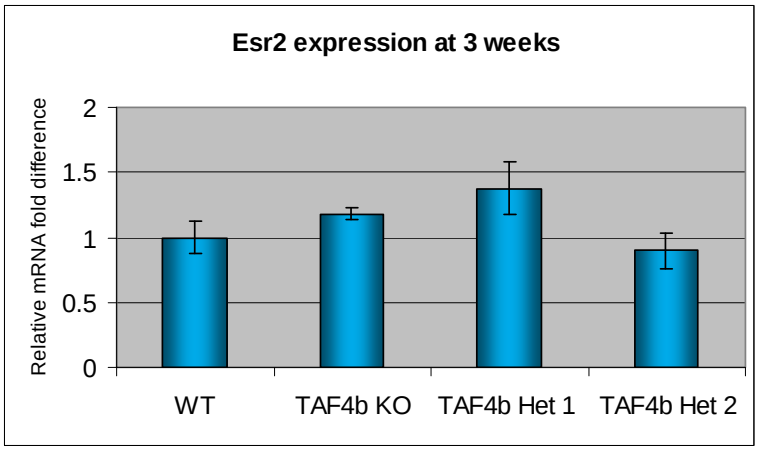


Figure 3. Granulosa cell proliferation defects in TAF4b-null ovaries. BrdU incorporation into the nuclei of follicular granulosa cells in 4 week-old TAF4b-heterozygous (left column) and null (right column) mice was assessed by immunolabeling. (A-B) Mice stimulated with 5 IU PMSG for 24 hours. (C-D) Mice stimulated with 1 μg/g estrogen for 24 hours. (E) Quantification of percent positive nuclei in follicles in the experiments in (A- D). Average values from two independent experiments are presented; error bars represent s.e.m.



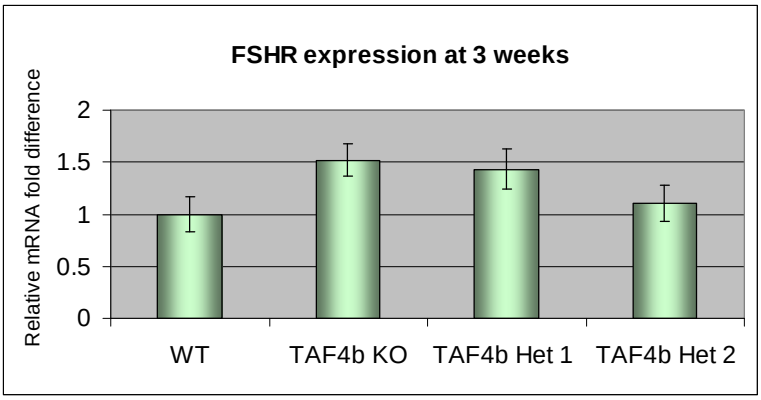


Figure 4. ERβ and FSHR expression in TAF4b-null and heterozygous ovaries. 3 week-old TAF4b-null and heterozygous littermates were injected with estrogen, ovaries were harvested after 24 hours and ERβ (Esr2) and FSH receptor (FSHR) levels were measuring by qRT-PCR. Data were normalized to 18S rRNA. Error bars represent standard deviation.

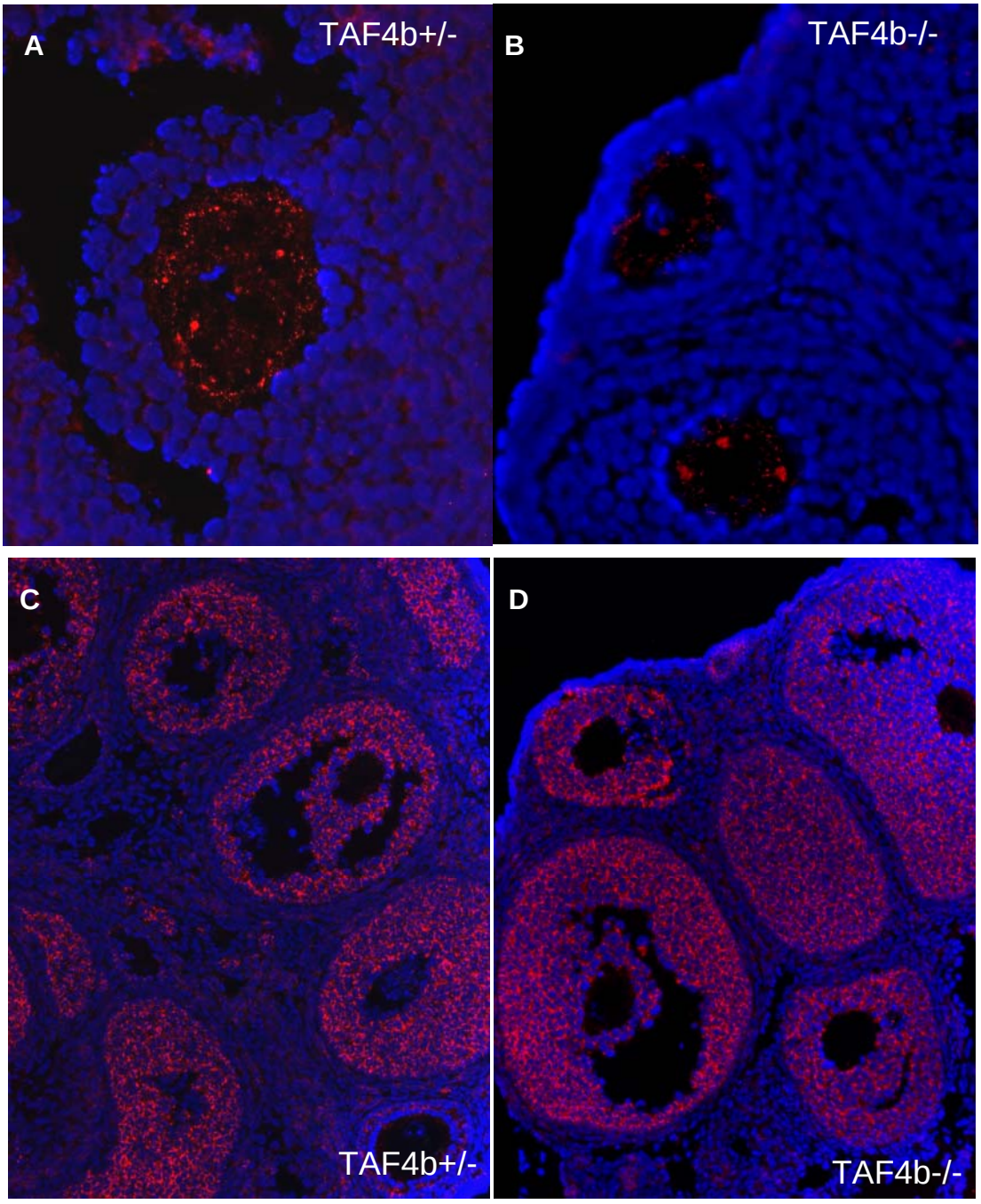
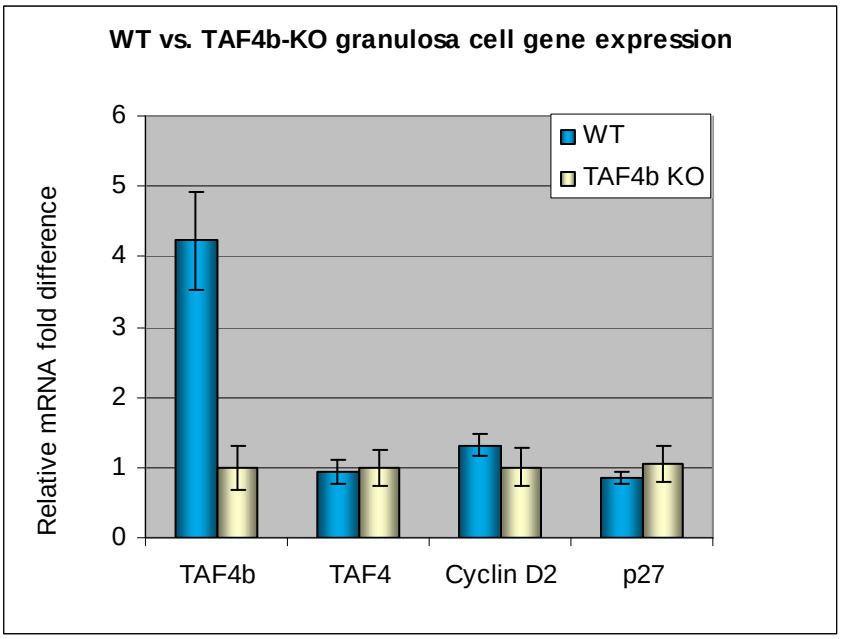


Figure 5. Connexin localization in TAF4b-null and heterozygous ovaries. Immnuofluorescent detection of (A-B) connexin 37 and (C-D) connexin 43 in (A,C) 4 week-old TAF4b heterozygote and (B,D) TAF4b-null ovarian follicles. Red= connexin, Blue= DAPI.

A



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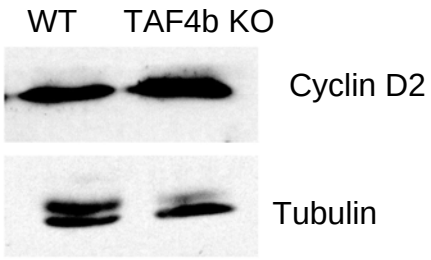
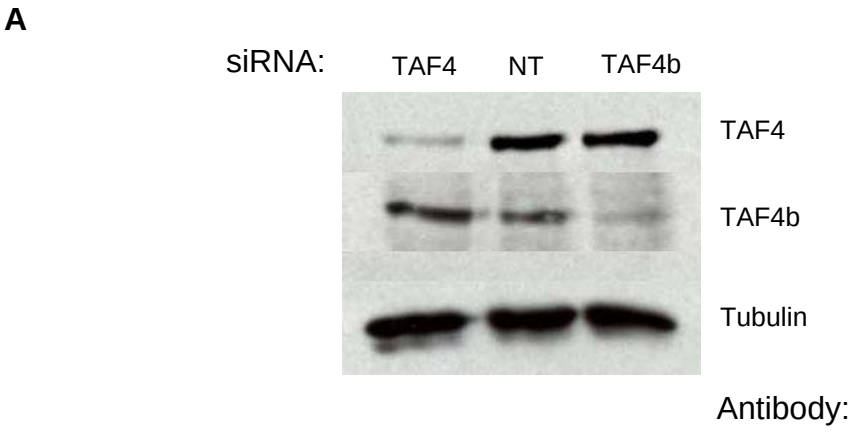


Figure 6. Gene expression in TAF4b-null and wildtype juvenile ovaries. (A) Granulosa cells were isolated from 4-week old TAF4b-null (KO) and wildtype (WT) mice. Levels of TAF4b, TAF4, Cyclin D2 and p27 were determined using qRT-PCR. Data were normalized to L-17. Error bars represent standard deviation. (B) Western blot detection of cyclin D2 in total ovary protein from 4 week old TAF4b-null and wildtype littermates. Tubulin serves as a loading control.



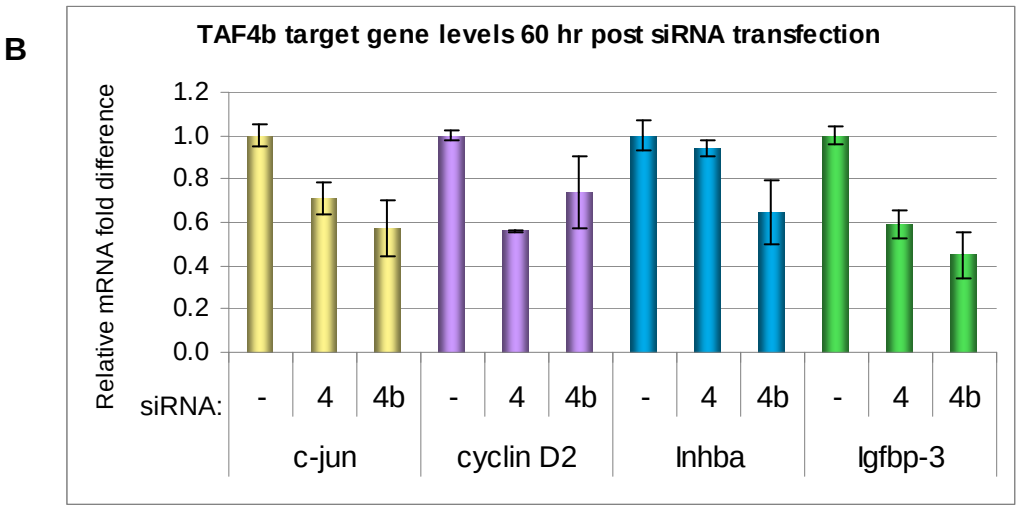


Figure 7. siRNA mediated knockdown of TAF4 and TAF4b reduces target gene expression. (A) Western blot of NT-1 cells following siRNA knockdown of TAF4 and TAF4b at 60 hour post-transfection. NT- non-targeting siRNA. (B) C-jun, cyclin D2, inhba and Igfbp-3 expression 60 hours post-transfection as determined by qRT-PCR. Data were normalized to 18S rRNA. Non-targeting value was set to 1. Error bars represent standard deviation.

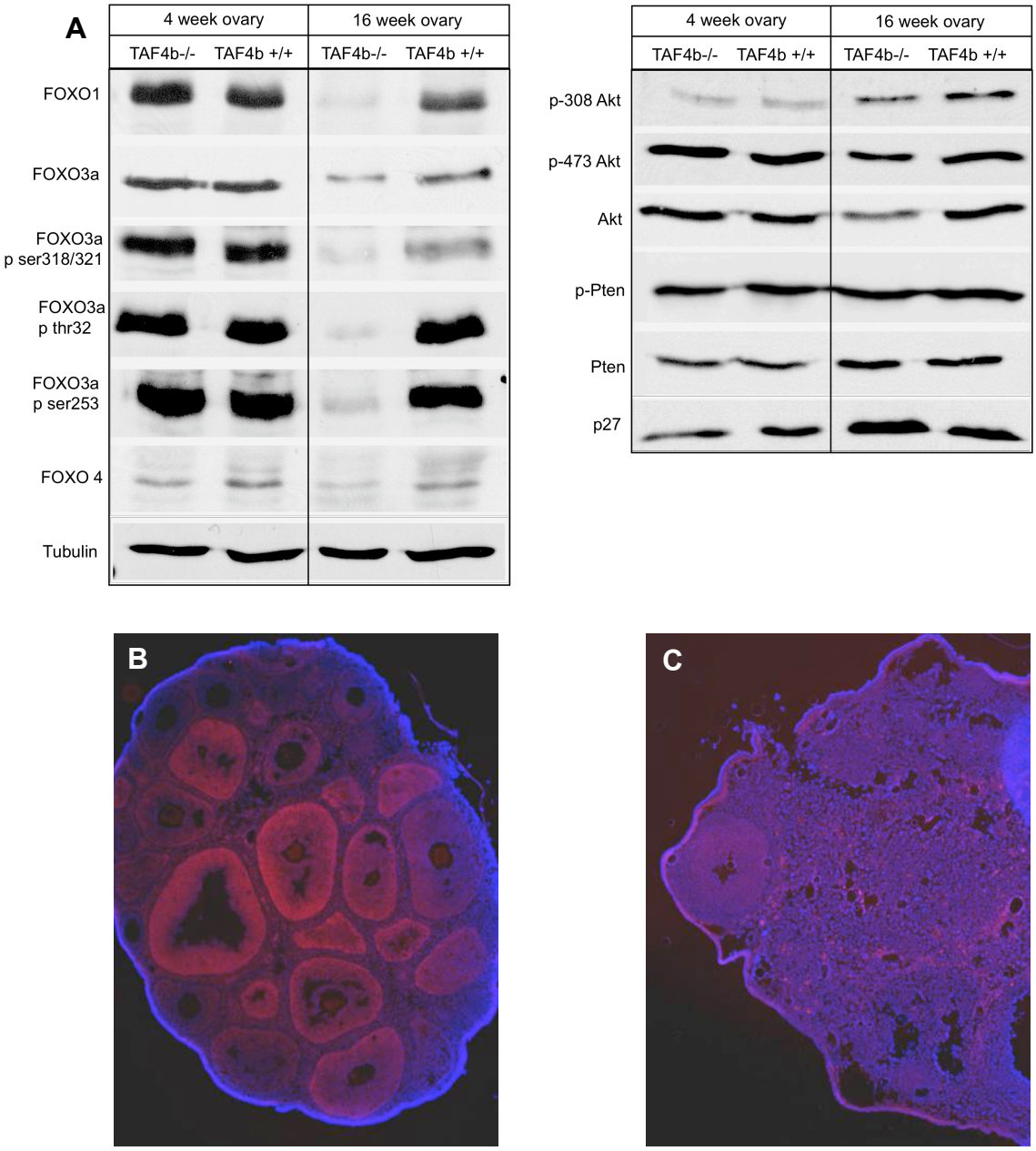


Figure 8. Age-dependent reduction of key ovarian regulatory proteins in TAF4b-null ovaries. (A) Western blot analysis was used to examine protein levels in the Foxo and Pten/Akt pathway using whole ovary extracts collected from 4 and 16 week-old TAF4b-null (TAF4b -/-) and wildtype (TAF4b +/+) ovaries. An equivalent amount of whole ovary protein extract was loaded per lane, confirmed by tubulin level. Immunofluorescent detection of Foxo1 protein in (B) 4 and (C) 16 week-old TAF4b-null ovarian follicles. Red= Foxo1, Blue= DAPI.

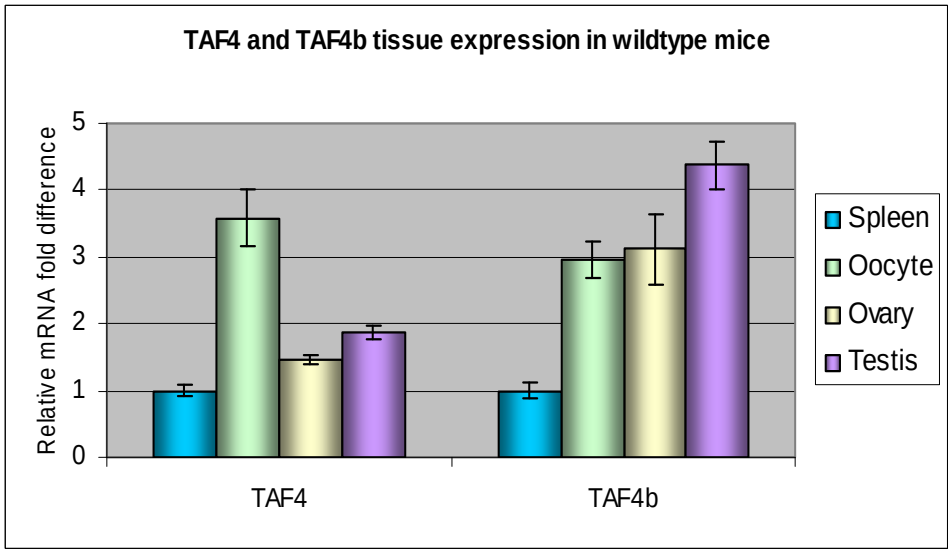


Figure 9. TAF4 and TAF4b tissue expression in wildtype mice. RNA was isolated from adult spleen, oocytes, total ovary and testis. Levels of TAF4 and TAF4b were determined using qRT-PCR. Data were normalized to 18S rRNA. Error bars represent standard deviation.

Chapter 3

Accelerated Ovarian Aging in the Absence of the Transcription Regulator TAF4b

Abstract

The mammalian ovary is unique in that its reproductive lifespan is limited by oocyte quantity and quality. Oocytes are recruited from a finite pool of primordial follicles that are usually exhausted from the ovary during mid-adult life. If regulation of this pool is perturbed, the reproductive capacity of the ovary is compromised. Previous characterization of TAF4b-deficient ovaries revealed several reproductive deficits that collectively result in infertility. However, the etiology of such fertility defects remains unknown. By assaying the estrous cycle, ovarian pathology and gene expression changes in young TAF4b-deficient female mice, we show that TAF4b-null females exhibit premature reproductive senescence. Here, we document the rapid decline of ovarian function in TAF4b-null mice that begins in early postnatal life and results in complete follicle depletion by sixteen weeks of age. To uncover differences in gene expression that may underlie accelerated ovarian aging, we compared genome-wide expression profiles of three week old, pre-pubescent TAF4b-null and wildtype ovaries. At three weeks of age, the decreased expression of specific genes in TAF4b-null ovaries is similar to that seen in aged ovaries suggesting TAF4b-null mice may be experiencing premature reproductive senescence. One significantly reduced transcript in the young TAF4b-null ovary encodes Mov10l1, a germline-specific RNA helicase that is related to the *Drosophila* RNA interference protein Armitage. We show here that Mov10l1 is expressed in the mouse ovary, declines normally with age and that its expression is dependent on TAF4b, linking TAF4b to the post-transcriptional control of ovarian gene expression.

Introduction

The regulation of aging in the mammalian ovary is a complex process and unique among diverse organ systems. A key factor garnering the length of female mammalian reproductive lifespan is the number of primordial follicles present in the ovary at birth and the maintenance of this finite pool during early postnatal life. Mouse models have been instrumental in our current understanding of the regulation of primordial follicle pool establishment and maintenance. For example, loss of the pro-apoptotic protein BAX in the mouse leads to reduced rates of atresia, a specialized form of oocyte cell death, increased numbers of primordial follicles and extension of ovarian lifespan into advanced chronological age [1]. Similarly, moderate caloric restriction during early female adulthood in mice leads to increased numbers of primordial follicles and delayed onset of reproductive senescence [2]. Alternately, Foxo3a-null mice prematurely deplete their oocyte stores by aberrantly activating follicle development, leading to infertility by 15 weeks of age [3]. Together, these data suggest that female reproductive senescence is greatly dependent on the regulation of the primordial follicle pool of the postnatal ovary.

In women, ovarian lifespan is not always directed by chronological age [4]. Often, young women seeking treatment at fertility clinics display characteristics of advanced ovarian age, such as reduced primordial follicle pool and elevated FSH. When the follicle reserve is low in women, oocyte quality is low regardless of chronological age, resulting in an increase in the number of chromosomal abnormalities and miscarriages [5]. Diminished oocyte quality in older women seems to be the major hurdle in conception. A previous study demonstrated that older women can carry and bear children just as well

as younger women if oocyte quality were controlled using oocytes donated by younger women [6]. Directed studies in mouse are in agreement with observations in humans of diminished oocyte quality with advanced age and have revealed that age-related increases in aneuploidy may be attributed to reduced transcript number of spindle assembly checkpoint genes [7]. Together, these data suggest that declining fertility with age reflects the declining quality of the oocyte and depleted follicle pool.

Recent gene expression studies in the mouse elucidated age-related gene expression changes in whole ovaries as well as oocyte-specific differences [8, 9]. Consistent with aging in other organs, aged ovaries showed increases in genes involved in immune functions and decreases in those associated with mitochondrial electron transport. However, the majority of transcript changes in the aged ovary were related to its organ-specific processes, a unique aspect of ovarian aging [9]. Molecular profiling in aged oocytes revealed decreased expression of genes involved in chromatin structure, genome stability and with RNA-helicase activity. Thus, the regulation of multiple aspects of gene expression might become increasingly impaired as oocytes age [8].

While many of the signaling factors controlling ovary function and reproductive aging are well known, less is known about the mechanisms of transcriptional integrators and effectors of specific oogenic transcriptional networks. Recent characterization of the general transcription machinery has revealed gonadal-specific or enriched variants of general transcription factors required for gametogenesis in diverse organisms [10-12]. TFIID is a large multi-protein complex which includes the TATA-box binding protein (TBP) and numerous TBP-associated factors (TAFs) which function collectively in core promoter recognition and gene co-activation [13-15]. One variant general transcription

factor that is essential for multiple aspects of mammalian gametogenesis is the gonadal-enriched TAF4b subunit of TFIID. In the mouse, TAF4b is required for proper female and male fertility. Although dispensable for the first round of spermatogenesis in the mouse testis, TAF4b is required for subsequent rounds and maintenance of spermatogenesis in the early adult [16]. In contrast, female mice that do not express TAF4b are completely infertile, owing to multiple defects of oogenesis [17-19]. TAF4b heterozygotes do not show an intermediate phenotype, but are comparable to wildtype with respect to fertility. Our most recent study of the ovarian defects associated with the loss of TAF4b revealed reduced numbers of primordial follicles, reduced granulosa cell proliferation and increased FSH levels [19]. An independent study also documented developmental defects in the oocytes derived from TAF4b-null mice [17]. Together, these studies suggest that rather than play a very specific role in female fertility, TAF4b executes several related functions that coordinate multiple aspects of female fertility, including those regulated by the somatic and oocytic compartments of the ovary.

The multifaceted nature of the female fertility defects in the TAF4b-null ovaries suggests that these mice are experiencing aspects of premature reproductive senescence. Many of the initial defects observed in the adult TAF4b-null ovary, such as decreased expression of several genes important in folliculogenesis, are not observed in the pre-pubescent ovary and cursory examination of the ovary suggests that TAF4b-null ovaries degenerate with age. To further examine the potential role of TAF4b in the transcriptional control of ovarian lifespan regulation, we compared the estrous cycle, ovarian development and gene expression profiles between pre-pubertal TAF4b-null ovaries and post-pubertal TAF4b-null ovaries. Here we show that in the absence of

TAF4b, female mice prematurely enter and exit the estrous cycle and develop hemorrhagic cysts compared to their corresponding wildtype counterparts. Candidate gene expression assays showed disruption of follistatin expression beginning between 2 and 3 weeks of age in the TAF4b-null ovary and pituitary. Genome wide whole ovary expression profiling at three weeks of age revealed the reduced expression of NACHT, leucine rich repeat and PYD containing (NALP) gene family members, oocyte-specific genes which have been shown to change their expression levels in aged ovaries [8], and Obox gene family members, which are exclusively expressed in germ cells [20]. Mad2, and Smc1β, important genes that show decreased expression with human maternal aging, are also decreased, as is the germline-specific RNA helicase, Mov10l1. These oocytic gene expression changes suggest that TAF4b-null ovaries are aging prematurely. Thus, as a whole, the TAF4b-null mice undergo premature reproductive senescence of the ovary due to the mis-expression of diverse gene programs within and outside of the ovary. These data suggest that deregulated TAF4b-dependent events may underlie premature reproductive senescence and infertility in women.

Results

Estrous cycle disruption in TAF4b-null mice

High FSH levels are often associated with irregular cycling in mouse models of infertility and in both natural menopause and premature ovarian failure in women. TAF4b-null mice exhibit high levels of FSH as early as 3 weeks of age, therefore, we hypothesized that TAF4b-null mice would be in a state of persistent estrus. Daily examination of cell type by vaginal smear cytology revealed that TAF4b-null females have irregular cycling;

a representative sample of disrupted cyclicity in TAF4b-null mice at 6 and 18 weeks of age is shown in Figure 1A. Initial cycling trials, lasting from 11 to 27 days, were completed with sexually mature mice from 6 weeks to 7 months of age. While all wildtype mice cycled appropriately, all TAF4b-null littermates were characterized as being in persistent estrus, exhibiting at least one ≥4 day period stalled in estrus. Persistent estrus usually accompanies the natural reproductive senescence occurring around 15 months (465 days) in wildtype C57BL/6 mice [2]. Surprisingly, 6 week-old TAF4b-null mice displayed relatively normal cyclicity during the first week of sample collection, while older TAF4b-null mice spent the vast majority of time in estrus with sporadic and brief re-entry into the estrous cycle. Upon discovering that the younger TAF4b-null mice could temporarily cycle normally, we set out to better characterize estrous cycling at the onset of estrous. We examined litters beginning at 3, 4 and 5 weeks of age and found that females did not remain in estrus for periods longer than 3 days until approximately 50±1.5 days of age (Figure 1B). Interestingly, we observed that TAF4b-null mice reproducibly entered estrous 4-6 days before their wildtype littermates. Onset of estrous for TAF4b-null mice is at pnd 24.5±1.3, for TAF4b-heterozygotes, pnd 28.2±1.1, and for wildtypes, pnd 29.8±0.5. Thus, defects in pre-pubertal TAF4b-null mice most likely leads to both accelerated activation and senescence of the TAF4b-deficient ovary. These data reveal a small window of normal cycling for TAF4b-null mice from an early onset at 24.5±1.3 days to a state of persistent estrus beginning at 50 ±1.5 days (Figure 1B). Contrary to our hypothesis, TAF4b-null females are able to traverse the estrous cycle despite elevated FSH very early in postnatal life, but quickly switch to a state of persistent estrus.

Histopathological examination of TAF4b-null ovaries

Disruption of the estrous cycle in TAF4b-null mice suggested that follicular development may be similarly deregulated. Our previous study included a comprehensive count of follicles stages at 3, 6 and 12 weeks of age and revealed that TAF4b-null ovaries contain reduced numbers of primordial follicles and increased numbers of atretic follicles at each time point, while recruitment to the primary follicle stage is comparable to wildtype. While we did not set out to uncover numerical follicle-stage data in the present study, we turned to histopathological examination of the TAF4b-null ovary to capture the precise timing of its rapid degeneration. To better understand the effects on the ovary during the progression from normal to irregular cycling, we harvested TAF4b-null and matched wildtype ovaries between 3 and 17 weeks of age for histological staining. At 3 weeks of age, before puberty and hormonal signaling has commenced in wildtype mice, TAF4b-null mice do not contain follicles of advanced stages, despite having elevated FSH levels and early entry to estrous (Figure 2A). Both wildtype and TAF4b-null ovaries contain relatively similar numbers of primary, but do not yet contain pre-ovulatory follicles. At 6 weeks of age, when the TAF4b-null mice are still cycling normally, the ovary appears normal and healthy, similar to its wildtype counterpart (Figure 2B). The larger appearance of the knockout ovary is a result of sectioning and at all time-points TAF4b-null ovaries are smaller than wildtype counterparts. At 8 weeks, TAF4b-null ovaries contain healthy antral follicles, which is interesting given that these mice are beginning to cycle irregularly (Figure 2C). Strikingly, only 1 week later at 9 weeks of age, the TAF4b-null ovaries display obvious signs of premature depletion of follicles (Figure 2D). By 10 weeks, the TAF4b-null ovary contains very few oocytes (Figure 2E). By 12

weeks, hemorrhagic cysts become evident (Figure 2F) and many fragmented oocytes are present in degenerating follicles (Figures 3A and B). The 17-week TAF4b-null ovary appears devoid of all developing follicles and oocytes (Figure 2G). In contrast, the wildtype ovary still contains some healthy follicles at one year of age and beyond (Figure 2H). We also observed an increased occurrence hemorrhagic cysts in the TAF4b-null mice (Figure 3A). TAF4b-null ovaries were observed with one, two or multiple cysts with the earliest identified at 12 weeks of age (Figure 2F). The ovaries collected from an aged wildtype mouse contained multiple cysts resembling those of the younger TAF4b-null animals (Figure 3A). Together, these data indicate that follicular development is relatively intact while the TAF4b-null mice are cycling normally and premature depletion of all follicles occurs rapidly by 17 weeks.

To examine the relative levels of TFIID components before and after TAF4b-null ovary degeneration, we harvested ovaries from 4 and 16 week-old littermates. We performed Western blot analysis on TAF4b-null and wildtype whole ovary lysate using antibodies against TAF4, TAF4b and TBP (Figure 4A). Loss of TAF4b does not change levels of the paralogous TAF4 subunit in the ovary, which remain constant between genotypes at both time points. However, TBP is slightly reduced at 16 weeks in the TAF4b-null ovary (Figure 4A). Interestingly, the level of TAF4b in the wildtype ovary is elevated at 4 weeks compared to 16 weeks. The elevated levels of TAF4b at 4 weeks in wildtype ovaries may suggest a greater need for TAF4b action at early stages of ovarian function, without which, leads to premature senescence. Alternatively, TAF4b may be regulated at the level of estrous-stage. 4 weeks of age is just at the onset of estrous in a wildtype mouse, whereas by 16 weeks of age, wildtype mice are cycling regularly. If

TAF4b protein levels are dynamic through the estrous cycle, this lower level may have been a result of examining the mouse in a phase where TAF4b is normally low. To this end, we collected granulosa cells at different points during the estrous cycle and found that neither TAF4b mRNA nor protein expression changes between estrus, diestrus and proestrus (Figures 4 B and C). Therefore, the reduction of TAF4b protein we see from 4 to 16 weeks of age is not a reflection of estrous stage.

Follistatin mRNA down-regulation in TAF4b-null ovary and pituitary

Although young TAF4b-null ovaries appear healthy, without a complete TFIID complex there may be underlying transcriptional deregulation from early time-points, which may ultimately lead to premature reproductive senescence. Previous data implicating TAF4b in control of follistatin expression, combined with high FSH levels, lead us to examine follistatin expression between TAF4b-null mice and wildtype littermates. To determine if TAF4b is necessary for proper follistatin expression, we performed qRT-PCR on ovary-derived RNA. At 3 weeks of age, while the TAF4b-null ovary still contains a large cohort of follicles and granulosa cells, follistatin mRNA levels are reduced 3-fold from wildtype (Figure 5A). At 7 months of age, when the TAF4b-null ovary contains no growing follicles, follistatin mRNA levels are decreased between 3- and 6-fold compared to wildtype and heterozygous littermates (Figure 5B). Together, these data suggest that loss of follistatin expression in the TAF4b-null ovary is not a result of loss of the granulosa cells in which follistatin is produced, but a specific gene expression mis-regulation associated with absence of TAF4b. However, follistatin does not always require TAF4b to achieve a normal expression level; at 2 weeks of age, follistatin levels in the TAF4b-null ovary are equivalent to those of its heterozygous littermates (Figure 5C).

In addition to granulosa cells of the ovary, the anterior pituitary also produces follistatin, which acts there as a paracrine signaling factor [21]. At 3 weeks of age, the TAF4b-null female expresses normal levels of key pituitary factors such as chorionic gonadotropin α subunit (Cga), gonadotropin releasing hormone receptor (Gnrhr), FSH subunit β (Fshb), LH subunit β (Lhb), Inhibin β B (Inhbb), and forkhead box protein l2 (Foxl2), but follistatin (Fst) levels are reduced 2-fold (Figure 6). Similarly to the ovary, pituitary expression of follistatin is not reduced at 2 weeks of age in the TAF4b-null mouse (data not shown). Follistatin, a critical component in the hypothalamic–pituitary-ovarian axis, appears to be de-regulated in the TAFb-null mouse both in the ovary and pituitary, and may contribute to elevated levels of FSH in TAF4b-null mice, but its normal expression levels at 2 weeks of age suggest that it is not at the core of the reproductive senescence in TAF4b-null mice.

Genome wide expression profiles of immature TAF4b-null ovaries

A candidate gene approach revealed surprisingly few gene expression changes at 3 weeks of age, thus it was likely we had missed some novel aspect of transcriptional deregulation within the pre-pubertal TAF4b-null ovary. To this end, we performed genome-wide expression profiling using microarrays with total RNA derived from the ovaries of 3 week-old wildtype, heterozygous and TAF4b-null mice in triplicate. By examining this time point, when the ovaries appear healthy, we hoped to obtain unbiased gene expression differences that result more directly from the absence of TAF4b and less from the complete loss of follicles. Of the 28,853 genes represented on the Affymetrix Mouse GeneST GeneChip, 1,488 non-redundant genes were present with significant ($P<0.05$) changes ranging from to 2.7 to 0.27 fold. (Complete microarray results have deposited in

the NCBI GEO under accession number GSE15228). Twenty-five genes were expressed in the TAF4b-null ovary at levels 1.5-fold greater than wildtype, while 60 genes were reduced 1.5-fold or more. Previous expression studies of aging in the ovary have found that the majority of gene changes are reductions [9]. Among the classes down-regulated are mitochondrial, those involved in oxidative stress, those involved in 'DNA metabolism' and oocyte-specific genes. Up-regulated genes tended to be involved in receptor binding and immune response/complement activation. The expression changes in TAF4b-null ovaries tended to be decreases in oocyte-specific genes. However, there was normal expression of many oocyte genes, indicating that there is not a global decrease in oocyte gene expression in our TAF4b-null samples (data not shown). The smaller size of TAF4b-null ovaries probably compensates for the lower number of oocytes, allowing for equivalent expression of oocyte genes between TAF4b-null and wildtype RNA samples when equal concentrations are measured. Functional annotation analysis of TAF4b-null vs. wildtype 3 week-old ovaries revealed that the main group of down-regulated genes in the TAF4b-null ovary belongs to the Homeobox family and related oocyte transcription factors called the Obox family (Table 1). A second down-regulated group includes gene products with nucleotide binding activity. Within this group are Moloney Leukemia virus 10-like 1 (Mov10l1) and multiple NALP genes. While the functions of Mov10l1 and the NALP genes have not been determined in the ovary, the NALPs were identified in screens for down-regulated factors in ovary aging [8], and preliminary data hint at important roles in oocyte development and aging [22]. Mov10l1 was found in male mice to be enriched in germ cells [23]. We confirmed expression changes identified in the microarray by qRT-PCR for several interesting genes

with roles in folliculogenesis or premature aging (Table 2). We found the largest reduction was of Mov10l1, with nearly a 6-fold reduction from wildtype and 4-fold from heterozygotes, followed by follistatin, and the NALP genes (Figure 7A). In contrast, Mov10l1's broadly expressed homolog, Mov10, was not significantly reduced in the TAF4b-null ovary. We confirmed that reduction of Mov10l1 was not an effect of early entry to estrous, because unlike follistatin, Mov10l1 levels are reduced at 2 weeks of age (Figure 7B). The majority of fold-changes in this study are subtle. By examining functional gene groups, we begin to build a picture of how numerous, small gene changes compound to exhibit the defects in TAF4b-null and the aging ovary. Together, these data indicate that TAF4b-deficient ovaries prematurely display gene expression changes characteristic of aging and are consistent with the complete infertility of the TAF4b-deficient female mice.

Reduction of meiosis-specific genes in TAF4b-null ovaries

Poor oocyte quality often leads to meiotic defects in older women, resulting in aneuploidy [24]. Several genes with confirmed roles in meiosis have been found to have down-regulated transcripts in oocytes of older women, likely contributing to the higher occurrence of chromosomal segregation abnormalities observed. We compared spindle checkpoint protein, Mad2 [25] and kinetochore protein, Bub1 [26] mRNA levels using qRT-PCR in TAF4b-null (n=2) and heterozygous (n=5) 2 week old ovaries and a 1-year old wildtype mouse ovary. Mad2 and Bub1 transcripts are reduced in TAF4b-null ovaries compared to their heterozygous siblings, and further reduced in aged, 1 year-old wildtype ovaries (Figure 8). Mad2 expression decreases 3.5 fold from the 2 week-old TAF4b heterozygous ovaries to the one year-old ovary, consistent with the trend in

published data [27]. Mad2 is also significantly (p=0.02) reduced in TAF4b-null littermate ovaries by about 30%. Bub1 shows a similar pattern of expression with an 8-fold reduction from 2 weeks to 1-year, but only shows a modest 10% reduction from heterozygote to TAF4b-null 2 week-old ovaries. Chromosomal segregation malfunctions can be caused by the premature separation of sister chromatids due to loss of cohesion. Structural Maintenance of Chromosome (SMC) proteins are integral components of the cohesin complex [28]. While SMC1α (Smc1a) is involved in mitosis, SMC1β (Smc1b) is meiosis-specific, and found to be down-regulated with age, contributing to aneuploidy [29, 30]. SMC1α expression is equivalent between 2 week-old TAF4b-null and heterozygotes, and 1-year old wildtype ovaries (Figure 8). Expression of SMC1β is very low in aged ovaries with an approximately 45 fold reduction from 2 week-old TAF4b heterozygote to 1-year old wildtype ovaries. Between 2 week-old ovaries, TAF4b-null expression is reduced 65% from heterozygotes. The reduction of SMC1β in 2 week-old null mice is highly significant ($p \leq 0.001$) and is one of the larger disparities between TAF4b genotypes we observe at such a young age, suggesting meiotic defects may contribute to the TAF4b-null phenotype.

Reduction of Mov10l1 in TAF4b-null ovaries

Mov10l1 is a very interesting gene that also may play a significant role in the TAF4b-null phenotype. While not much is known about the function of this putative RNA helicase, Mov10l1 is the mammalian homolog of the *Drosophila* protein, Armitage, which has been identified as a germline factor required for RNA interference (RNAi) in the ovary [31, 32]. In mammals, there is tissue-specific expression of Mov10l1 and its splice variants CHAMP and Csm. In the mouse testis, Mov10l1 is expressed as a full length

transcript of 25 exons [33, 34]. The heart-specific splice variants, CHAMP and Csm, lack the N-terminal coding sequence and the majority of these 2 proteins are composed only of the helicase domain (Figure 9A) [34, 35]. To follow up on the reduction of Mov10l1 in TAF4b-null ovaries, we set out to determine what splice variant is present in the mouse ovary. Using qRT-PCR with primer pairs independently amplifying the N and C terminal regions of Mov10l1, we analyzed wildtype mouse testis, heart and ovary (Figure 9B). The testis exhibited very high levels of expression of both the N and C termini, while heart expression was limited to the C-terminal domain, as previously shown [34]. Kidney and brain tissues exhibited negligible levels of both the N and C termini of Mov10l1 (data not shown). The 3 week-old ovary showed expression of both N and C termini, similar to the testis, but at much lower levels. Although well within the detection-limits of the assay, juvenile ovary expressed only about 1% of the testis Mov10l1 level. If Mov10l1 is restricted to germ cells within the gonad, lower expression in the ovary is not surprising. Unlike the testis, ovaries contain relatively few germ cells and do not have the ability to repopulate, thereby reducing their oocyte number with age. Indeed, we see an approximate 10-fold reduction in Mov10l1 from the 3 week-old to the 1 year-old wildtype ovary, consistent with germ cell reduction as ovaries age (Figure 9B). To determine if Mov10l1 expression is directly dependent on TAF4b we used an siRNA-mediated knockdown approach, *in vitro*. Since Mov10l1 is expressed in germ cells, we used the testis, germ-cell derived GC1 cell line to evaluate Mov10l1 expression levels upon TAF4b reduction. GC1 cells were transfected with siRNA targeting TAF4, TAF4b or a non-targeting control, followed by 60 hours of incubation, resulting in target protein levels reduction of approximately 75% (data not shown). It has been our experience that

knockdown of TAF4 causes a slight but reproducible increase in TAF4b transcript, which may be a compensatory mechanism (Figure 10A). While both knockdown of TAF4 and TAF4b were successful (Figures 10A and B), Mov10l1 transcript is elevated 75% upon knockdown of TAF4, and reduced 60% upon TAF4b knockdown, suggesting that its expression is enhanced by increased TAF4b levels and reduced by decreased TAF4b levels (Figure 10C). Mov10l1 reduction upon TAF4b knockdown is not the effect of global transcriptional down-regulation as TBP levels remain constant between siRNA targets (Figure 10D). Perhaps a Mov10l1 reduction in TAF4b-null oocytes reduces oocyte quality, leading to the appearance of advanced age.

Oocyte enrichment of Mov10l1

Data on the expression pattern of TAF4b within the ovary remains somewhat ambiguous. To suggest that loss of TAF4b leads to a reduction of Mov10l1 in the oocyte, we first need to determine the expression levels of TAF4b, Mov10l1 and their paralogs in purified populations of ovarian cell types. Using qRT-PCR we compared oocytes, granulosa cells and total ovarian RNA for expression of TAF4, TAF4b Mov10, and Mov10l1 (Figure 11). Here, we normalize to β-actin as a more appropriate control when comparing oocytes, which stockpile rRNA [36, 37]. Levels of Gdf9, an oocyte specific transcript, were measured to control for accurate separation of cell types and displayed a 30-fold enrichment over total ovary while detection in granulosa cells was less than 0.09 fold (data not shown). Similarly, TAF4b is detected in both oocytes and granulosa cells, with a slight, but neglible, enrichment in oocytes. TAF4 shows a significant enrichment in oocytes, yet is still expressed in granulosa cells. Mov10l1 has comparatively very little expression in granulosa cells, 0.06 fold of total ovary, while its expression in oocytes is

enriched 8-fold over total ovary. Unlike its paralog, Mov10 is present in granulosa cells with only a slight bias towards oocytes. Although this assay does not examine other ovarian cell types such as epithelial, luteal or thecal, we now have evidence that TAF4b is similarly expressed in both the granulosa cell and oocyte compartments of the adult mouse ovary. We also show for the first time that Mov10l1 is enriched in mammalian oocytes.

Discussion

The alterations in gene expression we observe in this study are consistent with the notion that TAF4b promotes the correct temporal expression of oocyte-specific genes required for the regulation of oocyte quality and ovarian lifespan. Unlike other organ systems the ovary seems to exhibit two distinct aging pathways, one of which is age-related oxidative damage, which can be accounted for by the buildup of ROS over time, and the other facet of ovarian aging is the reduction of oocyte-specific transcripts resulting in diminished oocyte quality. Women below the age of menopause often present with characteristics of older women and require assisted reproductive technology to conceive. It is possible that this subset of IVF patients are experiencing advanced ovarian senescence due to acceleration of normal ovarian aging pathways. TAF4b is a major regulator of multiple ovarian processes and disruption of this transcription factor could be an underlying cause of human infertility. In fact, a recent report has linked the integrity of TFIID and TAF4b expression to oocyte quality in women [38].

The reproductive defects observed in TAF4b-null female mice do not mimic the phenotype of any other transgenic mouse model of infertility. After the early onset of

ovary maturation, there is a brief period of normal cycling and ovaries from young TAF4b knockout mice look relatively healthy, even in the presence of elevated FSH levels. They contain a normal complement of follicles, albeit fewer in number, including some antral follicles that have undergone cumulus expansion. Thus, there does not appear to be one specific developmental block in TAF4b-null ovaries, but rather, a reduced capacity to transition nearly all stages of development in a timely fashion and an overall appearance of accelerated ovarian aging. The four major disruptions appear to be with 1) the number of primordial follicles in neonates, 2) the endocrine environment of the ovary beginning in pre-pubescence, 3) the quality of the mature oocyte and 4) granulosa cell proliferation and survival. An interesting aspect of the phenotype is that while confirmed TAF4b-target genes exist, TAF4b has not been absolutely required for their *in vivo* expression of any one gene at all time points.

To uncover gene regulation defects underlying the rapid deterioration of the TAF4b-null ovary, we first took a candidate gene approach, evaluating mRNA levels of genes previously found to be effected by TAF4b expression. Our initial microarray [18] compared TAF4b-null and wildtype ovaries from adult mice and attempts to recapitulate gene expression changes by qRT-PCR in younger mice was largely unsuccessful, as most genes are expressed at similar levels at 3 weeks of age. ChIP and RT-PCR data using cultured granulosa cell lines have identified TAF4b occupancy at target genes such as cyclin D2, c-Jun, Igfbp3 and follistatin [39, 40]. In many cases, genes that have shown differential regulation by TAF4b in cultured cells are expressed normally in the TAF4b-null mouse at younger time points. However, one gene that was found to be affected both in this study and in previous studies is follistatin, which is reduced 3-fold in the 3 week-

old TAF4b-deficient ovary [18, 39]. In fact, follistatin is up-regulated upon over-expression of TAF4b, and as determined by ChIP, TAF4b is bound to the follistatin promoter in cultured granulosa cells [39]. Follistatin is an important negative regulator of Activin thereby acting to suppress FSH release from the pituitary [41]. FSH is consistently elevated in TAF4b-null mice [17, 19]. The youngest reported FSH level measurement in the TAF4b-null mouse is 23 days, at which point FSH is elevated [17]. We show here that at 3 weeks of age and into adulthood follistatin is reduced in the TAF4b-null mouse. To see if follistatin levels were reduced at even younger ages, we examined 2 week old ovaries and pituitaries and found them equivalent between TAF4b-null and heterozygous mice. We do not currently know the FSH level of TAF4b-null 2 week old mice, but it is possible that between 2 and 3 weeks of age follistatin expression is disrupted leading to the phenotype of elevated FSH. We observed a premature entry of TAF4b-null mice into estrous which may be linked to disruption of the hypothalamic-pituitary-ovarian axis that also disrupts FSH and follistatin levels. Regulation of gene expression by TAF4b may be more temporal in nature than expected for a general transcription factor.

Young TAF4b-null mice display characteristics of premature ovarian aging such as persistent estrus, elevated FSH levels, a depleted primordial follicle pool, fragmented oocytes, poor oocyte quality, gene expression profiles consistent with advanced oocyte age, and hemorrhagic cysts. Cysts are not prevalent in the C57BL/6 strain, into which the TAF4b-null mice have been backcrossed [42]. In the wildtype MRL mouse strain, however, cysts are common, and the incidence of cyst formation increases with age [42]. The etiology of cyst formation is still speculative at this point but disruptions of genes

involved in diverse gonadal function increase their occurrence. Similar cysts have been reported in ovaries of granulosa cell-specific knockouts of follistatin [43], of mice lacking the tissue-bound isoform of follistatin [44], granulosa cell-specific knockouts of SF-1 [45], the *Cyp 19* (aromatase) knockout [46] and in mice with chronically high LH levels [47].

To uncover underlying gene expression changes that may lead to the rapid deterioration of the TAF4b-null ovary, we performed microarray analysis using 3 week-old ovaries from TAF4b-null females and their wildtype and heterozygous siblings. This genome-wide comparison revealed a number of oocyte-specific gene changes that may be indicative of advanced ovarian aging of the TAF4b-null ovary. The most dramatic transcript down-regulated in the knockout, 6-fold below that of wildtype, was Mov10l1. This putative RNA helicase has two splice variants, Csm and CHAMP, which are involved in heart development [34]. The full length Mov10l1, was identified as a testis-germ-cell-specific gene [33], but ESTs have since been identified in oocytes of mice and frogs [22, 48]. Mov10l1 shares a very high degree of homology with the *Drosophila* protein, Armitage [49]. Disruptions in Armitage lead to male and female sterility due to RISC maturation failure [32]. Here we show that the mouse ovary expresses Mov10l1 in an oocyte-enriched fashion, its expression is decreased in aged ovaries, presumably due to lower oocyte number, and that its expression is reduced by loss of TAF4b both *in vivo* and *in vitro*.

An interesting and unexpected finding was the reduction of a number of NALP (NACHT, leucine-rich repeat and PYD-containing), genes. The NALP family, identified in a screen for genes involved in oocyte aging [8], is composed of 14 members in humans

and 20 in mice, presumably stemming from several gene duplication events [50]. The NALP genes belong to a super-family of NOD-like receptors, which have roles in inflammation and innate immunity. Many of these genes are specifically expressed in the ovary, and loss-of-function in NALP5 (Mater), 4, 7, or 14 have been shown to cause female reproductive failure [50]. In the TAF4b-null ovary, Nalp4e, Nalp9c and Nalp4b were confirmed by RT-PCR to be reduced 3.11, 2.68 and 1.79-fold respectively. Interestingly, many of the NALP genes are clustered on chromosome 7 [50], very close to the location of the Obox genes, which are also down-regulated in the TAF4b-null ovary. Factor in the germline alpha (Figla), a critical oocyte transcription factor, also regulates the expression of several NALP genes [51]. Roles for NALPs in oocyte and pre-implantation development and innate immunity have been established, but functional data are scarce. Taken that expression of these genes is decreased with oocyte age and is regulated by two critical ovarian transcription factors, TAF4b and Figla, the NALP family may prove to be very interesting with respect to reproductive aging.

It is likely that the reduced number of primordial follicles in the TAF4b-null oocytes contributes to the very early follicle depletion observed by 17 weeks. TAF4b-null ovaries from pnd 1 mice contain the same number of primordial follicles as their fertile littermates, but by pnd 3, this number is reduced [17]. Interestingly, no increase in apoptosis is observed, leading us to question what has happened to these oocytes. A recent study on germ cell loss in the perinatal mouse has highlighted the role of autophagy in the initial wave of germ cell culling that takes place after birth [52]. Indeed, the microarray in this study showed a slight increase in autophagy genes (data not shown), which may be responsible for the lower number of germ cells in TAF4b-null

ovaries but this supposition requires much further work. Thus, a complex syndrome of ovarian defects occurring from shortly after birth through adulthood culminates in premature reproductive senescence and infertility in the absence of TAF4b.

The precise mechanism of how loss of TAF4b in mice leads to advanced ovarian age remains to be determined. The microarray performed in this study on pre-pubescent mice provides a new set of possible TAF4b-target genes involved in regulation of ovarian lifespan. Oocyte aging is not only regulated by factors within the germ cell, but is also profoundly influenced by the follicular microenvironment [53, 54]. Elucidating how TAF4b acts in granulosa cells, oocytes, or both, to maintain proper timing of ovarian events, will yield insight into normal regulation of ovarian aging, and may uncover new therapeutic opportunities for women who experience premature reproductive senescence and infertility.

Materials and Methods

Animals

Wildtype and TAF4b-null or heterozygous mice were generated by mating heterozygous TAF4b+/- male and female mice as previously described [18]. Offspring were genotyped by PCR analysis of tail-snip genomic DNA amplifying the region targeted by homologous recombination. Daily vaginal smear cytology was performed to determine estrous cycle stage. Mice were classified as in diestrus, proestrus, or estrus based on majority cell type present in vaginal sample. Determination of estrous cycle entry was deemed by the opening of the vagina and the presence of primary leukocytes in the sample. Samples from prepubescent mice were not taken if the vagina had not opened.

Cycle results from 4 wildtype, 4 TAF4b-heterozygous and 4 TAF4b-null mice were used to calculate onset of estrous. 4 or more consecutive days in estrus was used to define onset of persistent estrous. Cycle results from 3 TAF4b-null mice were used to calculate onset of persistent estrous. 21 mice, in total, were examined for estrous stage in this study. No mouse was cycled for more than 27 consecutive days. All animal protocols were reviewed and approved by Brown University Institutional Animal Care and Use Committee and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Ovarian histology

Ovaries were removed, cleaned of excess fat and bursal sac, and fixed in Bouin's solution overnight. Standard paraffin embedding, sectioning and Hematoxylin and Eosin staining were performed. Images were taken using the Aperio Scanscope CS.

Granulosa cell isolation

Ovaries from 8 week-old wildtype mice were removed, cleaned of excess fat and bursal sac and placed in DMEM F-12. Granulosa cells were liberated by pricking with 26 gauge needle. Resulting granulosa cell suspensions were filtered through 40 μm nylon filter to remove large tissue clumps and oocytes. Purified granulosa cells were washed in once PBS and re-suspended in Trizol (Bio-Rad) for RNA isolation or extraction buffer for protein isolation (0.2M KCl, 0.1M Tris, pH 8, 0.2mM EDTA, 0.1% NP-40, 10% glycerol, 1mM PMSF). Granulosa cells of 2 mice were combined per sample.

Oocyte isolation

Ovaries from 8 week-old wildtype mice were removed, cleaned of excess fat and bursal sac and placed in enzyme solution (0.1% collagenase type 3 (Worthington), 0.02% Dnase

type I (Worthington) and 0.1% trypsin (Invitrogen) in Hanks Balanced Salt Solution (Invitrogen)) and agitated for 30 minutes at 37°. Ovaries were dispersed using a 20G needle, the solution was diluted with DMEM-F12 and oocytes were collected. Oocytes were washed once with PBS and resuspended in Trizol reagent for subsequent RNA isolation. Ovaries of 3 mice, approximately 120 oocytes, were combined per sample.

Ovary isolation

Ovaries from 8 week-old wildtype mice were removed, cleaned of excess fat and bursal sac and Dounce homogenized in Trizol reagent for subsequent RNA isolation, or in extraction buffer for protein isolation. Ovaries of one mouse were used per sample.

Western blot analysis

Whole ovaries were Dounce homogenized and soluble protein extracted. Protein concentration was determined by Bradford assay and equivalent amounts of protein were loaded per lane. The following antibodies were used: BD Transduction Laboratories, TAF4 (TAF135) #612054, Novus Biologicals, β-Tubulin RB-9249-P, Abcam, TBP #ab818 and monoclonal TAF4b [55].

Microarray

Total RNA from 9, 3 week-old mice (3 wildtype, 3 TAF4b-heterozygous, 3 TAF4b-null) was obtained using Micro RNeasy kit (Qiagen) per manufacturer's instruction. RNA quality was checked using a Bioanalyzer, and concentration determined using a Nanodrop. 300 ng of each RNA sample was used in the Affymetrix Whole-transcript Sense Target Labeling Assay (Rev 3) followed by hybridization to a GeneChip® Mouse Gene 1.0 ST Array. 9 GeneChips were used to provide biological triplicates of each genotype. The Affymetrix Expression Console (v 1.1) was used to normalize data and

determine signal intensity (RMA-Sketch). Analysis was performed in Microsoft Excel and DAVID Bioinformatic software was used to identify gene expression pathways [56]. Complete microarray data can be obtained at the NCBI Gene Expression Omnibus under accession number GSE15228.

siRNA-mediated gene knockdown

TAF4 and TAF4b SMARTpool siRNAs and ON-TARGET*plus* Non-Targeting Pool (mouse) were purchased from Dharmacon and transfected into the mouse GC1 cell line using Dharmafect reagent 1. Optimization was performed per manufacturer's instructions. Cells were harvested at 60 hours post-transfection.

Quantitative RT-PCR

Total RNA was isolated from indicated samples using Trizol reagent (Bio-Rad). Glycogen was added at 2.5 μg/100 μl prior to isopropanol precipitation and RNA was isolated per the manufacturer's instructions. RNA concentration was determined using a Nanodrop. 20 μl of cDNA was prepared from approximately 500 ng of total RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to manufacturers directions, using a mix of oligo(dT) and random primers. Real-time PCR reactions were performed in triplicate using 2 μl cDNA template, ABI Power SYBR green PCR master-mix and gene specific primers in the ABI 7500 Real Time PCR System. Dissociation curves were generated and PCR products were run on an agarose gel to verify amplification of a single product. qPCR data were analyzed by the ΔΔCt method. Relative expression levels were normalized to 18S ribosomal RNA levels or β-actin as indicated to correct for equivalent total RNA levels.

Quantitative RT-PCR primers

	Forward (5'-3')	Reverse (5'-3')
Mov10	CCC GGT GAA AGC TAT GAA CT	GTT TCT GCT CCT TCT GCT CC
Mov10l1 C-term	CTC CAG GCT CTC CAA AAG TG	GGA CTA ATG GCC CAT AGG GT
Mov10l1 N-term	GCA CAG TGC TCT CCA GAT GT	AAC GGG GTG ATA GAG GAC AG
Follistatin	TAC TGT GTG ACC TGT AAT CGG A	TGA TAC ACT TTC CCT CAT AGG CT
NALP4e	GTT GGC ATC ATG TGT GCT CT	ACA GGC GGT TAT CAC AAA GC
NALP9c	TGA TAG TGA TGA TTT GGG AGC AG	AGT CCA ATT GAT GGC TGA AAA
Xlr5	CAC AGG GAA GGA AGC GTT AG	AAT CTC CCT TGC TGT CAT GG
NALP4b	GAG CTA AAA CTG CGA ATG GC	TCT GCT TCT GCT TAC ACA CCA
Mael	TGG TGT TTG AAG CGT ATG GA	ACA TGG CCA CAT CTA GGA GG
Ezh2	CAT CCA CGG CAG CCT TGT GA	TCA GGG TCA CAC TCT CGG ACA GC
TAF9b	CCA TAT GCA GGA CCC AGA CT	AGA TAC TGC TTG TGG GGG TG
Inhbb	CTC CGA GAT CAT CAG CTT TG	AGG TTC TGG TTG CCT TCA TT
Amh	GGG GAG ACT GGA GAA CAG C	AGA GCT CGG GCT CCC ATA
Foxl2	ACA ACA CCG GAG AAA CCA GAC	CGT AGA ACG GGA ACT TGG CTA
TAF4	ATC TCC ACT GTG CAG GCTT CC	GGT CAG CTG CCG TGC AAT A
Ccnd2	GCT CTG TGC GCT ACC GAC TT	AAT CAT CGA CGG CGG GT
c-Jun	CCC CTG TCC CCT ATC GAC AT	TTT TGC GCT TTC AAG GTT TTC
Igfbp3	CGG GAG ACA GAA TAC GGT CC	GCT TTC TGC CTT TGG AAG GG
Kit-L	ACA GCA GTA GCA GTA ATA GG	GAC TTG ACT GTT TCT TCT TCC
LHR	TCA GGA AAT TTG CCG AAG AAA GA	TCG TAA TCC CAG CCA CTG AGT T
TAF4b	GAT GTT ACT AAA GGC AGC CAA GAG T	CTG CTC TGG ATC TTC TTT ATT GGA G
Cga	GAC TTT ATT ATT CAG GGT TGC CCA	AGA AGC AAC AGC CCA TAC ACT G
Lhb	TGT CAA CGC AAC TCT GGC C	GGC AGT ACT CGG ACC ATG CT
Fshb	GAC TGC ACA GGA CGT AGC TGT TTA	CTG AGA TGG TGA TGT TGG TCA ATT
GnrhR	TTC ATC AAG ACC CAC GCA AA	GAG GTA GCG AAT GCG ACT GTC
Smc1a	AGG TGA TGG AGC AAT TAG GG	TTT TGT GTA GGC TGG CAG AG
Mad2l1	GAA ACG AGG GAA GAA TCA GG	TCT TCA TTG ATG GGG TTT TG
Smc1b	TGT GCT GGG ATG AGA AAG AG	TTC AGA TCT GTT TCC TTG CG
Bub1	GCT AGG ACC TTT GGA GAA CG	GCT AGA GTT TTG TTG CAG CG
18S rRNA	CCG CGG TTC TAT TTT GTT GG	GGC GCT CCC TCT TAA TCA TG

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Table 1. Functional characterization of decreased transcripts in TAF4b-null ovaries at 3 weeks of age.

Functional Groups Down-regulated in 3 week-old TAF4b-null Ovaries		
Genbank Accession#	Gene Name	Affymetrix fold reduction from WT
Group 1–Homeobox-related		
BC099488	Similar to oocyte specific homeobox 3	1.96
NR_003269	Obox 2	1.77
NM_175342	Cytoplasmic polyadenylated homeobox (Cphx)	1.68
NM_008759	Sebox	1.56
NM_145709	Obox 5	1.54
NM_145707	Obox 3	1.53
NM_011487	Signal transducer and activator of transcription 4	1.52
BC023444	One cut domain, family member 1, HNF-6	1.50
Group 2- Nucleotide binding proteins		
NM_001004194	NACHT, leucine rich repeat and PYD containing (NALP)4e	2.70
NM_031260	Moloney leukemia virus 10-like 1	2.22
NM_001042612	NALP9c	1.99
NM_145216	Rasl10a protein	1.81
NM_001048219	NALP9a	1.77
NM_145228	2'-5' oligoadenylate synthetase 1h	1.67
NM_009449	Tubulin, alpha 3	1.63
NM_007723	Cyclic nucleotide gated channel alpha 1	1.59
NM_029916	Ser thr kinase 31	1.55
NM_172481	NALP4b	1.52

Table 2. Fold changes of selected ovary transcripts between 3 week TAF4b-null and wildtype (WT) mice by microarray and qRT-PCR.

	3 week-old TAF4b-null ovary mRNA	
	Fold of WT	
Gene	Microarray	RT-PCR
Mov10l1	0.45	0.17
Fst	0.62	0.32
NALP4e	0.35	0.32
NALP9c	0.50	0.37
Xlr5b	0.52	0.40
NALPb	0.66	0.56
Mael	0.62	0.70
Ezh2	0.83	0.72
Mov10	1.07	0.72
TAF9b	0.75	0.79
Inhbb	0.60	0.94
Amh	0.92	1.00
Foxl2	1.07	1.03
TAF4	0.93	1.05
Ccnd2	0.89	1.05
c-Jun	1.09	1.29
Igfbp-3	1.25	1.39
Kitl	1.23	1.61
Lhr	1.47	1.64

Microarray fold differences are an average of 3 data points. RT-PCR fold differences are the results from amplification of pooled cDNA from the RNA used in the microarray.
Mov10l1- Murine leukemia virus10-like1, Fst- Follistatin, Xlr5b- X-linked lymphocyte-regulated 5b, Inhbb- Inhibin B, Mael- Maelstrom, Ezh2- Enhancer of zeste homolog 2, Mov10- Murine leukemia virus10, Amh- Anti-mullerian hormone, Foxl2- Forkhead box protein l2, Ccnd2- Cyclin D2, Igfbp-3- Insulin-like growth factor binding protein-3, Kitl- Kit-Ligand, Lhr- Luteinizing hormone receptor.

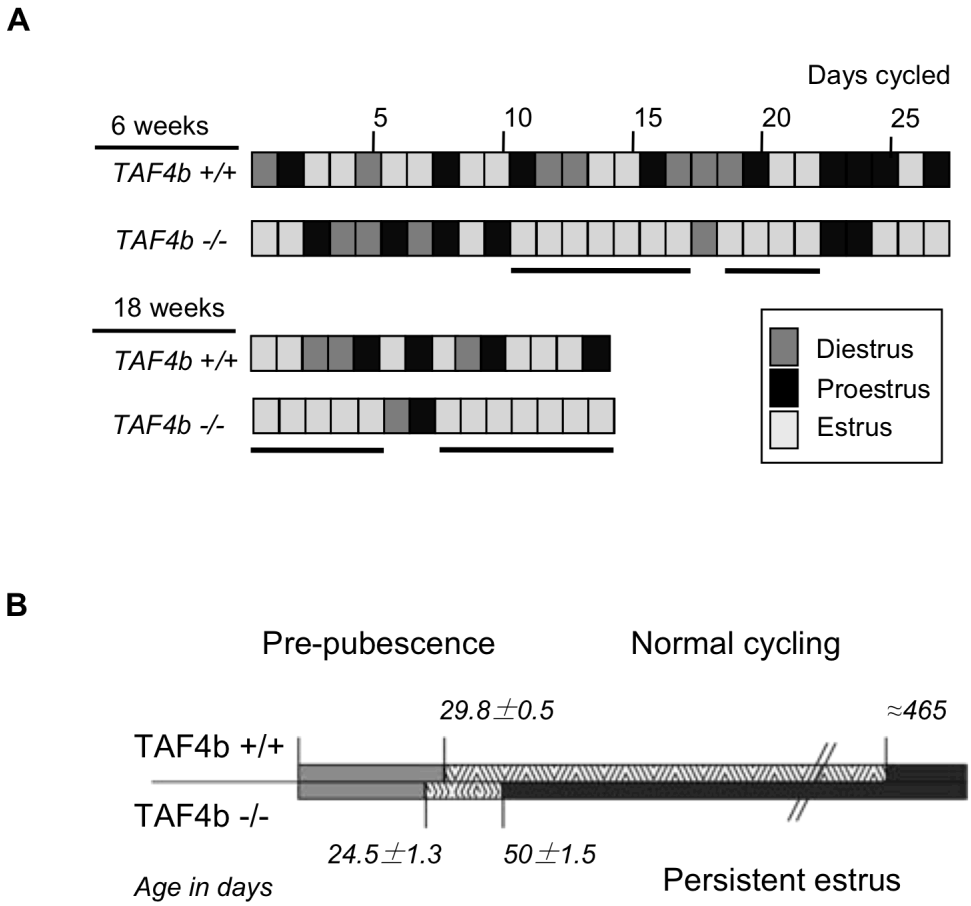


Figure 1. Estrous cycle disruption in TAF4b null mice. Vaginal smear cytology was performed daily to assess the estrous stage of TAF4b-null (TAF4b -/-) and their wildype (TAF4b +/+) littermates. (A) Representative samples of wildtype and TAF4b-null mice, cycled daily, starting at 6 and 18 weeks of age. Each block indicates the stage of the mouse for that day. Days indicating persistent estrus are underlined. (B) Summary of wildtype (TAF4b +/+) and TAF4b-null (TAF4b -/-) estrous cyclicity with average age at onset of puberty at 24.5±1.3 days for TAF4b-null mice (n=4) and 29.8 days for wildtype mice (n=4). TAF4b-null mice (n=3) enter persistent estrus at 50 days ±1.5 while wildtype mice do not until about 465 days (15 months) [2].

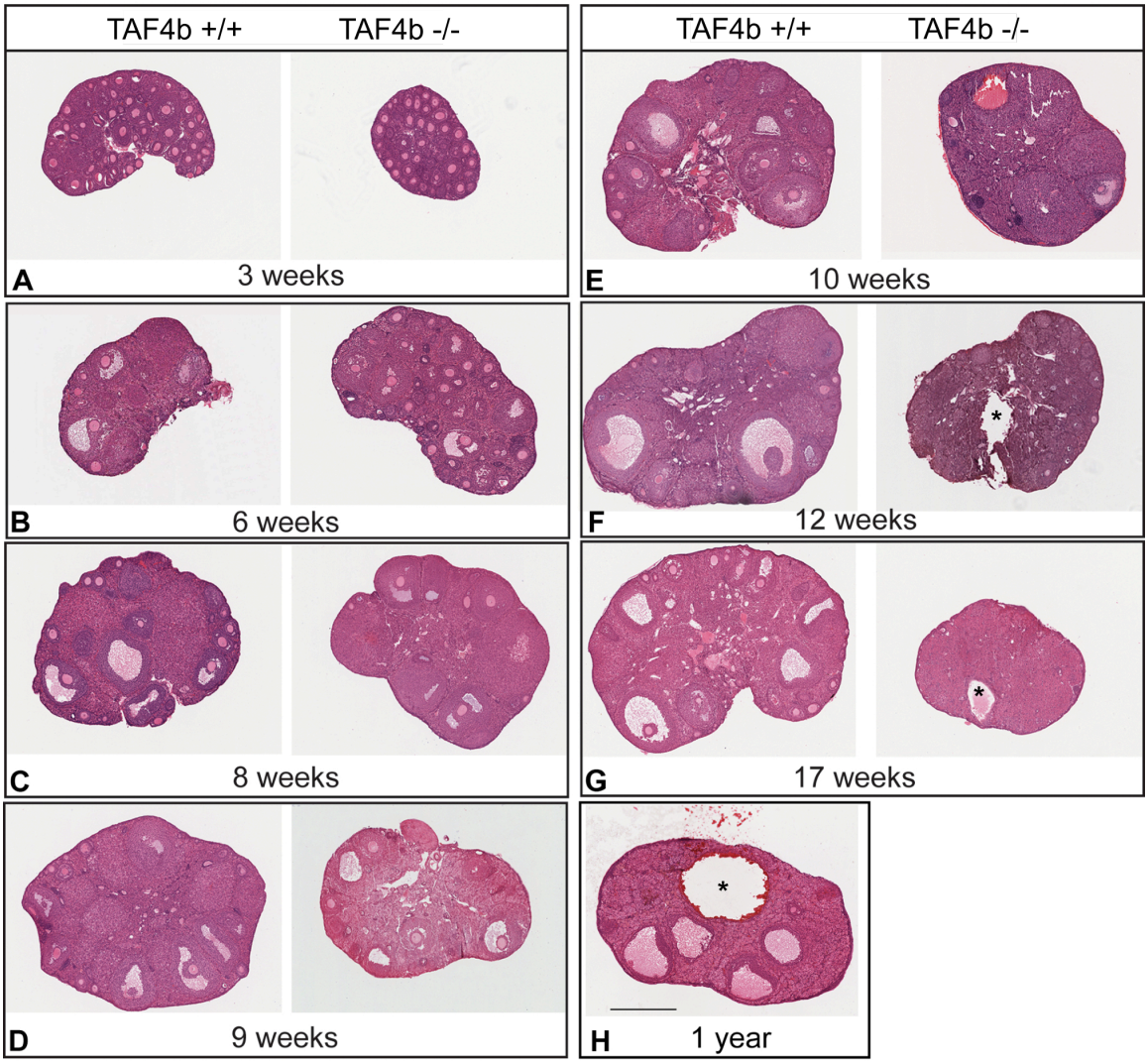


Figure 2. Progressive follicle loss in TAF4b-null ovaries. Gross ovarian histology using hematoxylin & eosin staining of Bouin's fixed ovaries at (A) 3, (B) 6, (C) 8, (D) 9, (E) 10, (F) 12 and (G) 17 weeks of age from TAF4b-null (TAF4b -/-) mice and their wildtype (TAF4b +/+) littermates showing the progressive and rapid loss of maturing oocytes. (H) 1year-old wildtype ovary serves as a reference for normal aging in the C57/BL6 mouse line and shows the presence of both developing follicles and a hemorrhagic cyst. Bar = 500 μm. *Asterisk= hemorrhagic cyst.

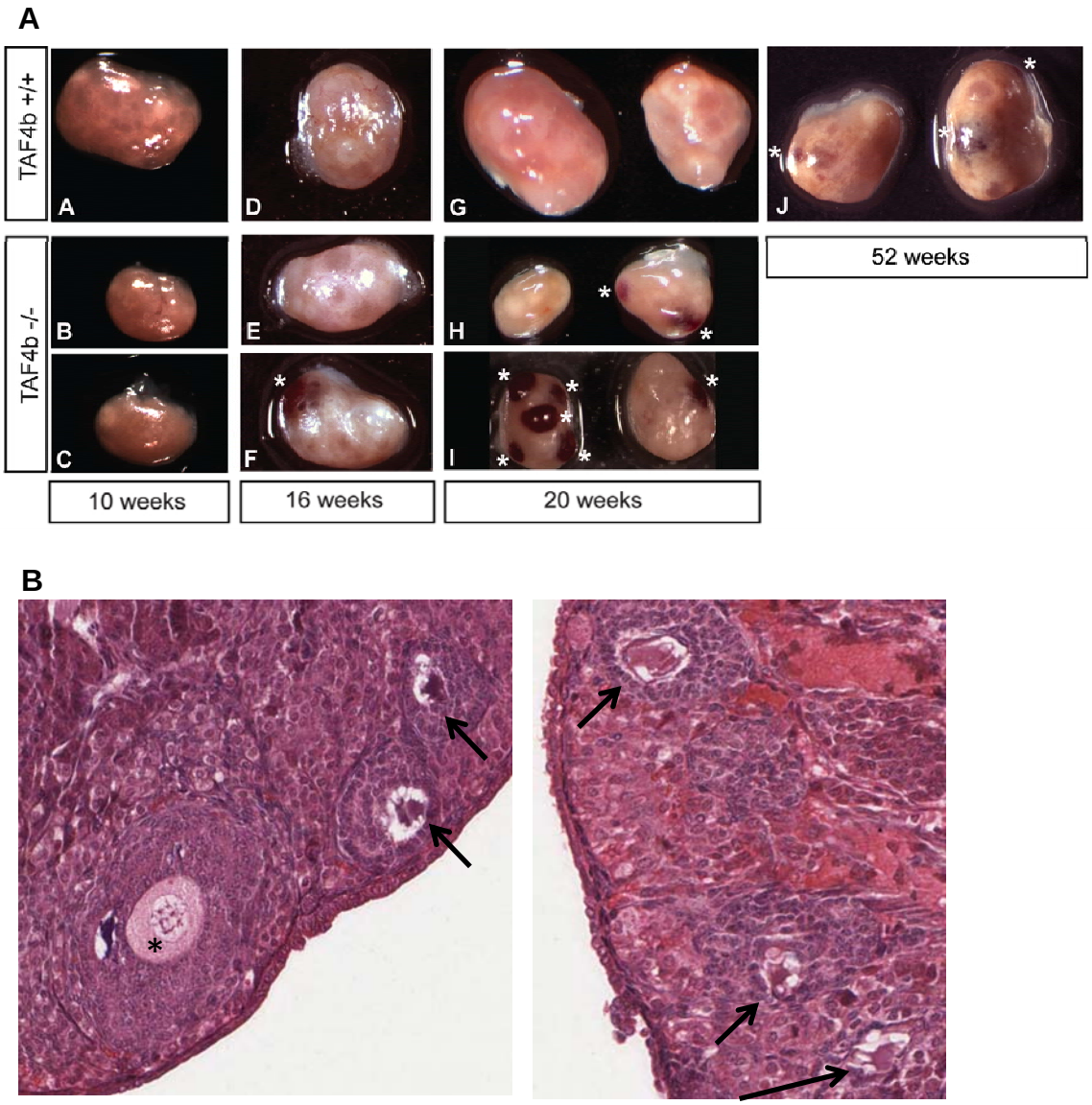


Figure 3. Cyst formation and oocyte fragmentation in TAF4b-null ovaries. (A) TAF4b-null ovaries display hemorrhagic cysts. The occurrence of hemorrhagic cysts in TAF4b-null (TAF4b -/-) ovaries increases with age. At 10 weeks of age cysts are not present, but are observed by 16 weeks and regularly seen at 20 weeks and beyond. Cysts are not common in fertile littermates, but are apparent in the older, 1year-old wildtype. *Asterisk = hemorrhagic cyst. (B) Fragmented oocytes present in degenerating follicles of 12 week TAF4b-null ovaries. Hematoxylin & Eosin staining of ovary sections derived from 12 week-old TAF4b-null ovaries. Fragmenting oocyte denoted by arrow. Healthy oocyte denoted by asterisk *.

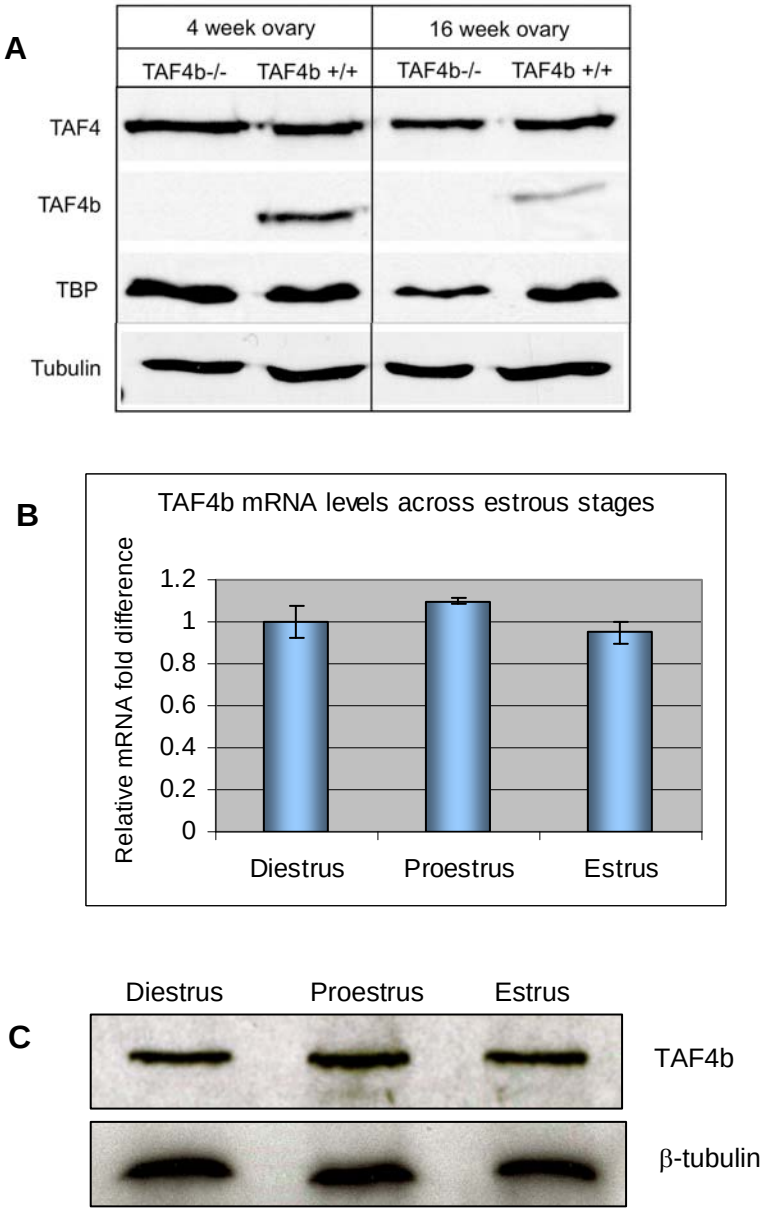


Figure 4. TAF4b expression changes with age but not throughout estrous cycle. (A) Western blot analysis shows protein levels of TAF4, TAF4b and TBP at 4 and 16 weeks of age in TAF4b-null and wildtype total ovary lysates. TAF4b (B) mRNA and (C) protein levels were assessed in wildtype mice at diestrus, proestrus, and estrus. Cycle stage was determined using vaginal smear cytology, granulosa cells were harvested and RNA or protein was isolated. (A) Total RNA was subject to qRT-PCR using TAF4b-specific primers and normalized to to18S rRNA. Error bars represent standard deviation. (B) Granulosa cell protein was subject to Western blot analysis. β-tubulin was used as a loading control.

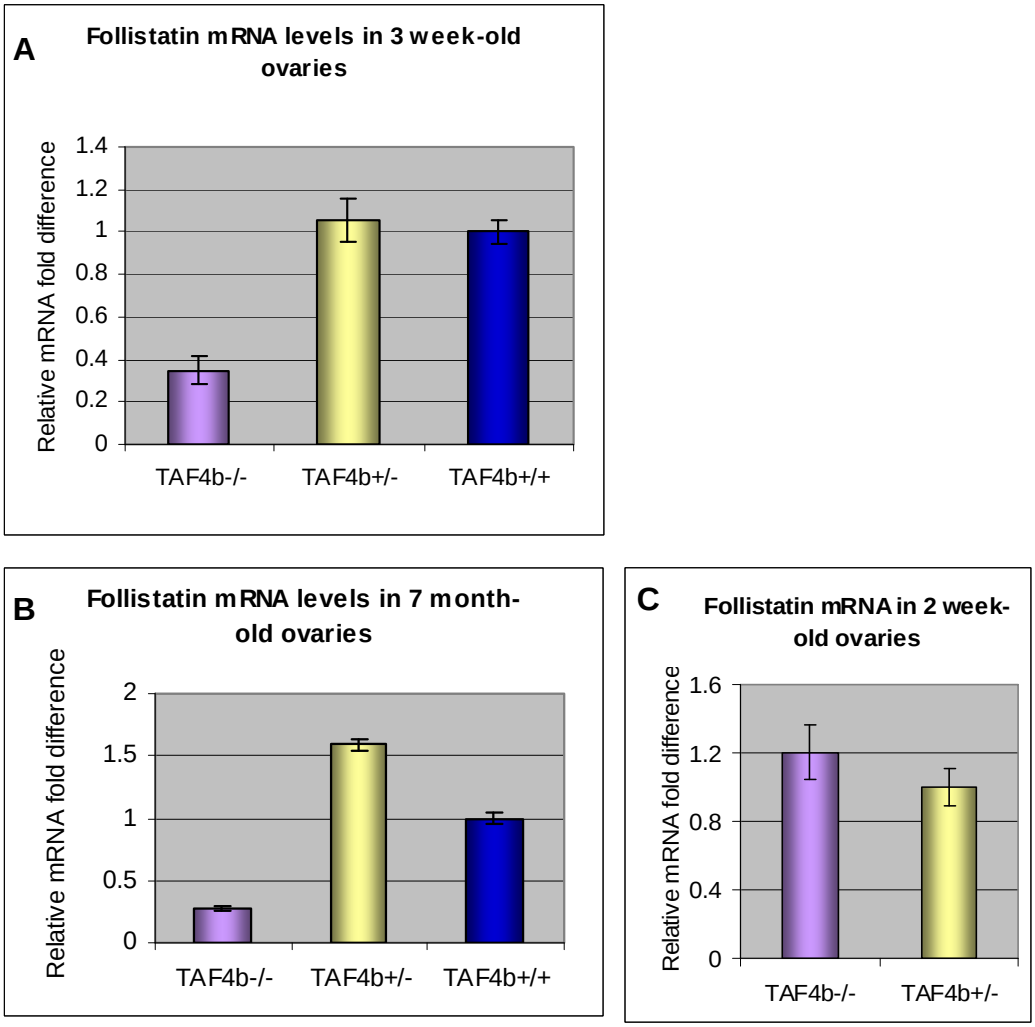


Figure 5. Defects in TAF4b-null follistatin ovary expression. RNA samples were obtained from (A) 3 week-old ovaries, (B) 7 month-old ovaries and (C) 2 week old ovaries. qRT-PCR was used to compare gene expression of TAF4b-null ovaries to wildtype and/or heterozygous littermates. Data was normalized to 18S rRNA present in cDNA sample. Relative mRNA fold difference is displayed after setting wildtype or heterozygote value to 1. Error bars in (A) and (B) represent standard deviation. Error bars in (C) represent s.e.m. TAF4b +/- n=2, TAF4b -/- n=5.

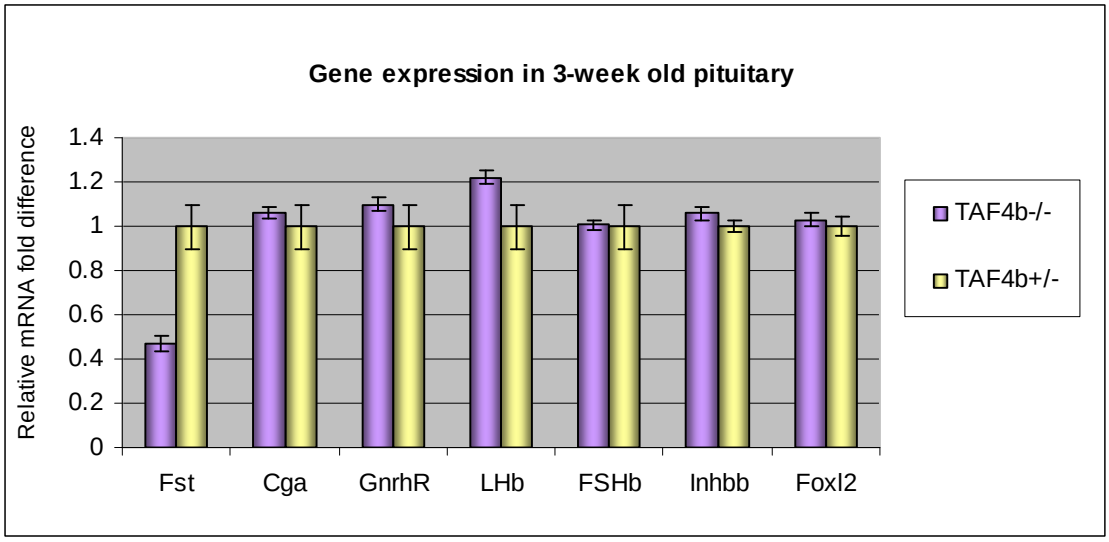


Figure 6. Defects in TAF4b-null follistatin pituitary expression. RNA samples were obtained from 3 week old pituitaries of TAF4b-null and heterozygous mice. qRT-PCR was used to compare mRNA expression of Fst, Follistatin, Cga, chorionic gonadotropin, GnrhR, gonadotropin releasing hormone receptor, Fshb Follicle-stimulating hormone subunit β, LHb luteinizing hormone subunit β, Inhbb Inhibin β B, and Foxl2 Forkhead box protein l2. Data was normalized to 18S rRNA present in cDNA sample. Relative mRNA fold difference is displayed after setting heterozygote value to 1. Error bars represent standard deviation.

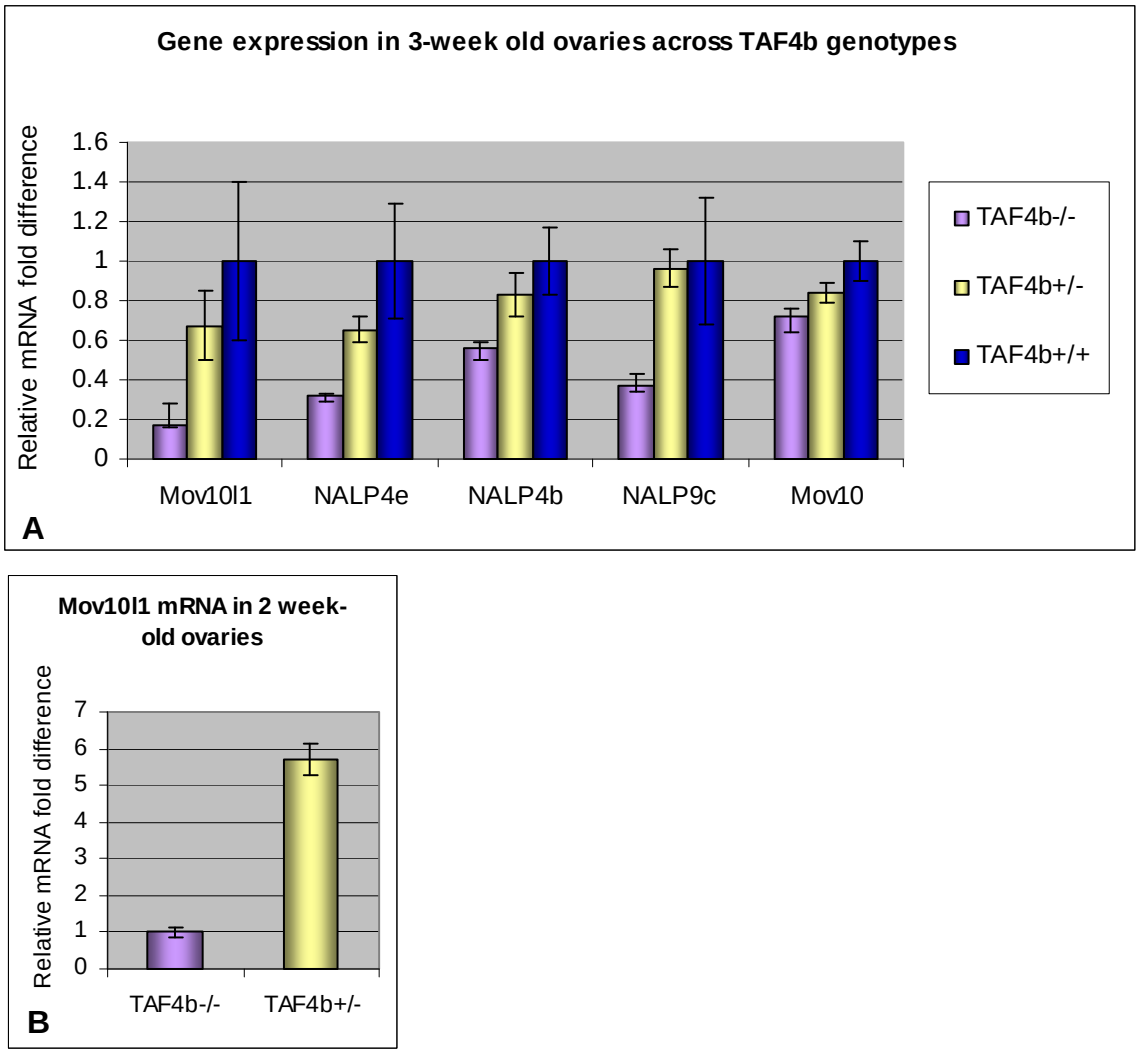


Figure 7. Ovarian gene expression changes in TAF4b-null ovaries vs. wildtype and heterozygous littermates. Ovaries from (A) 3 week-old TAF4b-/-, TAF4b+/- and TAF4b+/+ littermates, and (B) 2 week-old TAF4b-/- and TAF4b+/- littermates were used in qRT-PCR using gene specific primers. Data was normalized to 18S rRNA present in cDNA sample. Relative mRNA fold differences is displayed after setting the wildtype sample to 1 in (A), and TAF4b-null sample to 1 in (B). Error bars in (A) represent standard deviation. Error bars in (B) represent s.em. TAF4b +/- n=2, TAF4b -/- n=5.

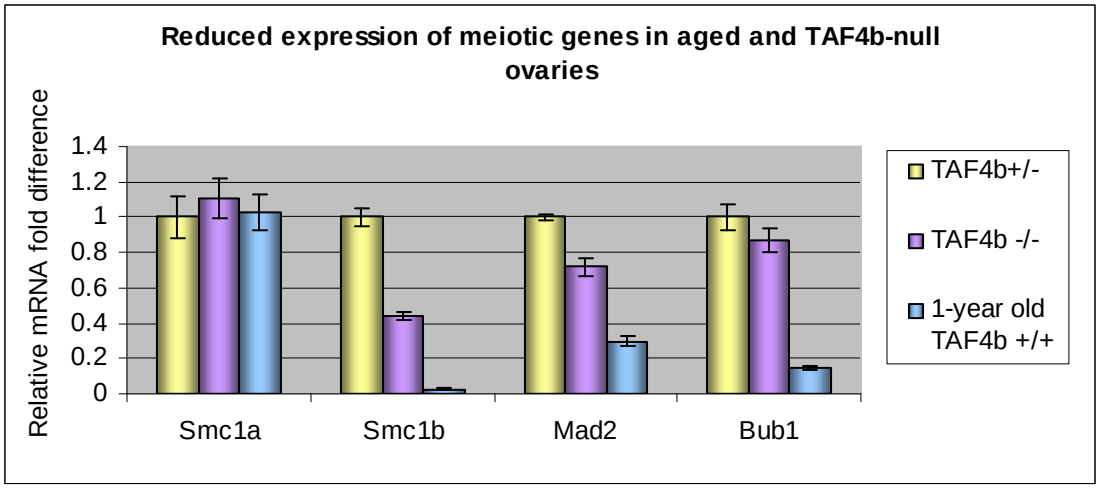
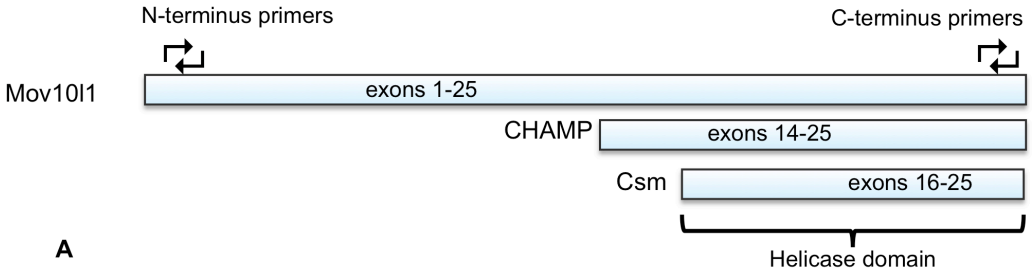


Figure 8. Reduced expression of meiotic genes in aged and TAF4b-null ovaries. Ovaries from 2 week-old TAF4b-null mice (n=5), heterozygous littermates (n=2) and a 1-year old wildtype mice were used in qRT-PCR using gene specific primers for Smc1a Smc1b, Mad2 and Bub1. Data was normalized to 18S rRNA present in cDNA sample. Relative mRNA fold difference is displayed after setting 2 week-old TAF4b+/- sample to 1. Error bars represent s.e.m.



A

	Percentage Mov10l1 expression vs. testis	
	N-terminus	C-terminus
Adult testis	100± 14.5	100± 16.8
Adult heart	0.0012 ± 0.00008	34.8 ± 0.44
3 week-old ovary	1.37 ± 0.046	0.96 ± 0.11
1 year-old ovary	0.11 ± 0.008	NT

B

Figure 9. Full length Mov10l1 is expressed in ovary and testis. (A) Full length Mov10l1 and its splice variants CHAMP and Csm. Of the 25 exons comprising full-length Mov10l1, CHAMP is missing the first 14, while Csm is missing the first 16 and contains only the helicase domain. (B) Primers specific to the N or C terminus of Mov10l1, as diagrammed in (A), were used in qRT-PCR to determine splice variation between adult testis, adult heart, juvenile ovary (3 weeks-old) and aged ovary (1 year-old). Data was normalized to 18S rRNA present in cDNA sample. With the highest expression of both the N and C terminus, adult testis was set to 100% expression. Percentage expression vs. testis is indicated with 95% confidence intervals. NT=not tested.

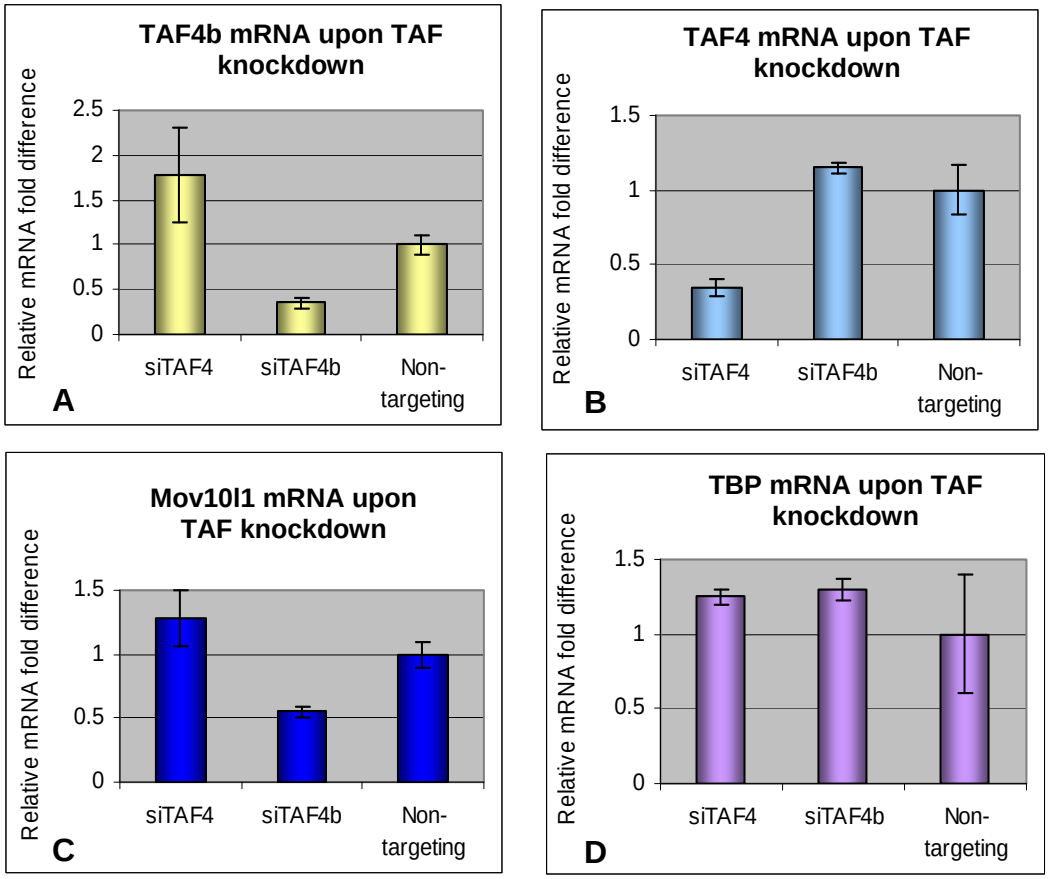


Figure 10. Mov10l1 is reduced upon TAF4b knockdown. GC1 cells were transfected with siRNA targeting TAF4, TAF4b, or with a non-targeting RNA. At 60 hours post-transfection cells were harvested, RNA was isolated and cDNA was made. qRT-PCR was performed using gene specific primers for TAF4b, TAF4, Mov10l1 and TBP. Data was normalized to 18S rRNA present in cDNA sample. Relative mRNA fold difference is displayed after setting non-targeting sample to 1. Error bars represent s.e.m. from 2 independent trials.

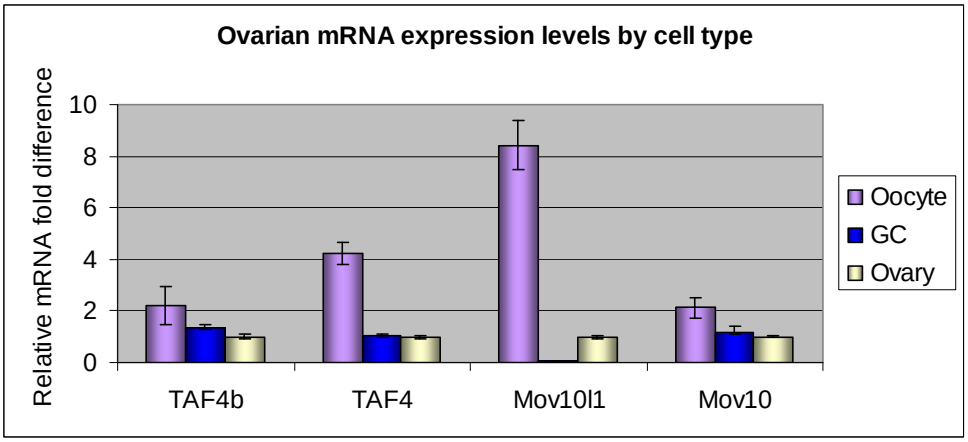


Figure 11. Ovarian mRNA expression levels by cell type. Oocyte, granulosa cell or total ovary RNA was collected from 8-week-old wildtype mice. qRT-PCR using gene specific primers for TAF4b, TAF4, Mov10l1 and Mov10. Data was normalized to β-actin present in cDNA sample. Relative mRNA fold difference is displayed after setting ovary sample to 1. Error bars represent s.e.m. Oocyte n=2, GC n=3, Ovary n=3.

Chapter 4

Dynamics of TAF4b in liver proliferation

Abstract

TAF4b-null mice are defective in several gonadal processes, including specialized programs of proliferation. Several lines of evidence from work done in B-cells, testis, ovary and MEFs point towars a role for TAF4b in promoting cell cycle progression. To test TAF4b's involvement in proliferation we utilized the regenerative capacity of the liver. Preliminary data suggested that TAF4b is induced during regenerative growth following partial hepatectomy. Here, we perform partial hepatectomies in wildtype and TAF4b-null mice to assay TAF4b's involvement in the rapid cell proliferation during regenerative growth. Although TAF4b is induced following hepatectomy, ultimately, TAF4b is not required for liver mass restoration, and there does not appear to be a lag in the cell cycle in TAF4b-null mice compared to wildtype. There is also no apparent compensation by TAF4 in TAF4b-null livers. Interestingly, TAF4b shows induction during regenerative growth, but low expression during fetal liver growth which differs from the expression trends of other TAFs.

Introduction

Based upon the expression profiles of TAF4b, we hypothesized that TAF4b may have an important role outside of the gonad in promoting cellular proliferation in multiple cell types. Although cellular proliferation during embryogenesis is not impaired in TAF4b-null mice, the adult tissue distribution of TAF4b protein in the mouse lead us to question its specific involvement in cell proliferation. Although there are high levels of TAF4b protein in the gonads, there is also enrichment in the spleen. One commonality between the testis, ovary and spleen is their high rate of proliferation compared to fully differentiated tissues like the heart and liver. It has been demonstrated that TAF4b protein is elevated following lipo-polysaccharide (LPS) -stimulated proliferation in B-cells, and expression of TAF4b is highest in the proliferating spermatogonia of the testis and granulosa cells of the ovary [1-3]. A study in mouse embryonic fibroblasts (MEFs) revealed that in cells that contained TFIID complexes with only TAF4b, and not its paralog TAF4, serum-dependent growth regulation is lost and the cells proliferate uncontrollably [4]. The current view in the field is that a balance exists between TAF4 and TAF4b containing TFIID complexes, with TAF4b promoting a proliferative response. Therefore, incorporation of TAF4b into TFIID not only directs the transcription of specific genes, but may also prevent the repressive growth effects of TAF4.

The liver provides an excellent *in vivo* system to study the dynamics of TAF4b expression between quiescent and proliferating states in the adult mouse. Unlike other organs, the liver possesses the unique ability to undergo regenerative growth after mass removal by injury or surgery. Functioning to remove toxins from the body, the liver must

be able to handle exposure to harmful compounds and has likely developed its regenerative capacity as a survival mechanism [5]. In the standard model of partial hepatectomy in rodent, approximately 68% of the liver is removed by resecting the left, left medial and right medial lobes [6]. Mass regeneration occurs not by regenerating the missing lobes, but by enlargement of remaining right and caudate lobes until the original mass of the liver has been restored. The response to hepatectomy is an immediate and synchronized event, with approximately 95% of the hepatocytes exiting G0 to re-enter the cell cycle [5, 7, 8]. Although the adult liver contains the multi-potent Oval cell, stem cells do not account for the proliferation that occurs following hepatectomy, rather, hepatocyte replication is responsible for regeneration [9].

Following partial hepatectomy in the mouse, DNA synthesis begins at about 32-36 hours, representing the initial and synchronous wave of replication in hepatocytes. The non-parenchymal (non-hepatic) liver cells, such as Kupffer and billiary epithelial cells, undergo replication as well but not until several hours after the hepatocytes [5]. Gene activation following hepatectomy can be transient or sustained throughout the proliferative response, which can take up to 2-weeks in rodents [5, 6].

Interestingly, the genes involved in compensatory growth following hepatectomy are different than those responsible for proliferation during embryogenesis. Near the end of gestation, embryonic livers undergo rapid hepatocyte proliferation that leads to a tripling in liver mass [10]. TAF4b-null mice do not seem to have defects in somatic tissues during development, suggesting that the program of fetal liver proliferation is not impaired.

Results

TAF4b protein is upregulated in rat following hepatectomy

To determine if TAF4b may be involved in adult liver regeneration we consulted with Dr. Phil Gruppuso, who generously provided liver nuclear extracts from sham and post-hepatectomy rats in which 2/3 of the liver had been removed. Since our TAF4b monoclonal antibody reacts with the rat protein, we performed western blot analysis on samples from 24 and 48 hour post-hepatectomy and corresponding sham samples (Figure 1). TAF4 was present at similar levels between sham and hepatectomized livers, while TAF4b showed a lower basal level which was elevated approximately two-fold at both 24 and 48 hours post-hepatectomy.

With the preliminary finding that TAF4b is preferentially up-regulated following hepatectomy, we prepared to extend this study into mice. After pilot hepatectomy trials we determined that a 2/3 removal of the liver caused an unacceptable mortality rate and that a 50% removal would better suit our study. To accomplish a 50% removal we excised the left and left medial lobes while leaving the right, right medial and caudate lobes to recover. To reduce the number of animals used in this study we chose not to use a sham control for every hepatectomy, instead comparing gene expression between the left lobe and the right lobe of individual animals. The left and right liver lobes are very similar in size and growth rates so after assaying a number of transcript levels between a removed left lobe and sham right and left lobes, we decided to use the left liver lobe as a baseline for expression levels compared to a regenerated right lobe [11]. We performed hepatectomies on wildtype mice allowing 12 or 24 hours of regeneration to determine if the results from rat extend to mouse. Unfortunately, we could not use Western blot

analysis to detect TAF4 or TAF4b in the protein extracts obtained from these samples. The antibody we used was made against rat TAF4b, which may explain its ability to detect the low levels of TAF4b in the rat but not the mouse. After numerous, failed extraction procedures to concentrate TAF4b protein, we decided to use qRT-PCR, an assay with increased sensitivity, to determine if there were changes in TAF4 or TAF4b expression following hepatectomy. At 12 hours post-hepatectomy, TAF4b mRNA levels in the regenerating right lobes increased by 85% and TAF4 levels had increased by 50%, but by 24 hours levels had returned to those of unstimulated, left lobes (Figure 2).

TAF4b mRNA peaks at 5 hours post-hepatectomy in mouse

To gain a better understanding of the dynamics of TAF4b and TAF4 mRNA expression following hepatectomy we performed several 50% hepatectomies in adult wildtype mice and determined the extent of induction at specific time points (Figure 3). It appears that TAF4b is up-regulated as soon as 2 hours following the surgery, reaching a maximum of just under 3-fold induction at 5 hours, and then returning to basal level. Interestingly, this peak of expression is not seen for TAF4 with the time-points we examined, suggesting that these related TFIID components are differentially regulated during liver regeneration.

Gene expression in response to hepatectomy is not dependent on TAF4b

To see if TAF4b plays a role in gene expression following hepatectomy we assayed expression levels of several mRNAs in wildtype and TAF4b-null mice at 5 hours post-hepatectomy (Figure 4). Not only was the TAF4b-null liver able to up-regulate genes involved in regeneration as well as the wildtype, the response in some cases was more robust. Cyclin D1 and E1 are known to be up-regulated in the regenerating liver and do

not rely on TAF4b for expression [10, 12]. Interestingly, previously reported TAF4b-target genes are also able to be up-regulated in response to hepatectomy. The expression of insulin-like growth-factor-binding protein-3 (Igfbp-3) has been shown to be affected by TAF4b levels in granulosa cells [13, 14], as have the TGF-β family members inhibin α (*inha*), inhibin βA (*inhba*) and inhibin βB (*inhbb*) [13]. The data from the current study suggests that loss of TAF4b in the liver does not affect expression of Igfbp3, inhibin βA or inhibin βB (Figure 4).

Another TAF4b target gene, cyclin D2 is involved in hepatocyte proliferation in the fetal liver but not in the adult [10]. To gain insight as to how the TAF4b-null livers develop, we assayed levels of TAF4, TAF4b and cyclin D2 in embryonic-day 17.5 and quiescent adult liver (Figure 5). As expected, adult cyclin D2 levels were much lower than those of the fetal liver. Cyclin D2 expression in the TAF4b-null liver was equivalent to its heterozygous and wildtype littermates. Interestingly, TAF4 mRNA, which was not up-regulated during liver regeneration was elevated in the fetal liver compared to the adult. TAF4b, however, was expressed at the same, or slightly lower, level between fetal liver and quiescent adult liver, indicating that there is differential regulation of general transcription factors between fetal and postnatal regenerative growth.

TAFs are differentially regulated between developmental and regenerative liver proliferation

To see if there is a trend in the expression of general transcription factors between fetal and adult liver we assayed the level of other components of RNA Polymerase II (Pol II) transcriptional machinery in fetal liver and in adult quiescent and proliferating liver (Figure 6). Med26, a mediator complex component, TAF1, TAF3 and TAF4 in fetal liver

were expressed approximately 2-fold over adult quiescent liver and did not have elevated expression at 3 hours following hepatectomy, although they may have been elevated at different time points. TBP was expressed equivalently between fetal and quiescent liver and had elevated expression at 3 hours following hepatectomy. TAF4b levels appeared approximately 60% greater in quiescent liver than in fetal liver, and its expression was doubled at 3-hours post-hepatectomy. These data suggest that like the cyclins there are different requirements for general transcriptional machinery between fetal and regenerative hepatocyte proliferation.

Liver regeneration is not compromised in TAF4b-null mice

Since we found TAF4b to be induced upon liver regeneration, we tested if TAF4b is required for this process to proceed normally. To address this question we harvested regenerating livers from TAF4b-null, heterozygous and wildtype mice following hepatectomy as well as sham controls. At 6 days post-hepatectomy, which is about mid-way through the regeneration process, livers were harvested and individual lobes weighed (Figure 7). Left lobes were removed during the hepatectomy, leaving the right lobes to undergo compensatory proliferation. In most cases TAF4b-null livers were able to regenerate mass in the remaining right, right medial and caudate lobes equivalent to TAF4b-heterozygotes and wildtype mice. These data suggest that TAF4b is not absolutely required for proliferation of hepatocytes during regenerative growth.

The early induction of TAF4b following hepatectomy may point to an early role in liver regeneration and perhaps any defects or delays in proliferation may have been overcome by 6 days. To determine if TAF4b-null mice have any delay or defect in early proliferative events following hepatectomy, we tested BrdU incorporation between

wildtype and TAF4b-null livers. In the mouse, BrdU incorporation following hepatectomy reaches a peak after approximately 40 hours [15]. We determined that BrdU incorporation between wildtype and TAF4b-null livers at 48 hours post-hepatectomy was equivalent (Figure 8). Scoring over 1,000 cells in each the wildtype and TAF4b-null liver, we found they differed by only a fraction of a percent, indicating that TAF4b is not required for cell division after hepatectomy.

Discussion

TAF4b does not appear to have an essential role in hepatocyte proliferation following partial hepatectomy in the mouse, refuting the hypothesis that TAF4b is required for this proliferative response. The factors involved in liver regeneration are largely redundant and deletion of a single gene does not usually result in complete loss of hepatic regeneration, but may cause a delay in mass gain [5]. We have shown that while TAF4b is upregulated following partial hepatectomy, liver mass recovery is not impaired by the end of regeneration. Induction of DNA synthesis in the TAF4b-null liver also proceeds at the same rate and with the same timing as wildtype livers, suggesting that TAF4b is not required for a timely response to liver removal. It is likely that other components of TFIID compensate for loss of TAF4b, allowing for appropriate transcription. The most probable candidate for compensation is TAF4. We analyzed expression of TAF4 and TAF4b at several time-points following hepatectomy and observed that while TAF4b expression was highly elevated by 5 hours, TAF4 levels remained fairly constant. It is possible that TAF4 expression could have been transiently upregulated at a time-point we did not examine. If TAF4 were compensating in TAF4b-null livers, we might expect to

see an induction of TAF4 following hepatectomy in TAF4b-null mice. Although we did not perform a sufficient number of hepatecomies in TAF4b-null mice to confidently say that TAF4 is never induced, we did show that TAF4 is not significantly upregulated at the time-points we would expect to see elevated TAF4b levels. Another unexpected finding was the relatively normal expression of TAF4b target genes in the regenerating TAF4b-null liver. Although previous studies have found that TAF4b influences the expression of Igfbp-3, inhibin α and inhibin βB, we have shown that TAF4b is not required for expression of these genes in the liver [13, 14]. It is very likely that TAF4b is involved in expression of these genes in different systems and perhaps the compensation that may be occurring in the liver is not available in other cell types.

It appears that the requirement for components of TFIID differs between programs of proliferation in the fetal and adult liver, much like the cyclin D family. Cyclin D2 and D3 are highly expressed during fetal liver proliferation, while cyclin D1 is not expressed during fetal proliferation, but highly expressed following hepatectomy in the mouse [10] . In other systems TAF4b is bound to the cyclin D2 promoter, but again, we found that its expression is not altered in TAF4b-null developing livers, and no elevation of TAF4 is observed to explain possible compensation. In comparing levels of other Pol II transcriptional machinery, we found that Med26, TAF1, 3 and 4 are expressed at higher levels in the fetal proliferating liver than the adult resting or 3-hour post-hepatectomy livers. TAF4b and TBP, in contrast, are not higher in fetal liver and have elevated levels following hepatectomy. The differential expression of the so-called 'general' transcription factors that make up TFIID provides evidence that the cell can

alter the composition of this complex in response to hepatectomy, introducing another level of transcriptional regulation.

TAF4b's up-regulation, but non-essential, role following hepatectomy is very similar to what is observed in LPS-stimulated B-cell proliferation. Originally identified in a B-cell line, TAF4b was thought to have a cell-type specific function there, but following extensive testing, it was found to have a non-essential role in B-lymphocyte proliferation or development [1, 16]. There is a very interesting parallel between TFIID component dynamics during regenerative proliferation in the liver and LPS-stimulated proliferation in B-cells. In both cases, a robust induction of TAF4b and TBP is observed while TAF4 and TAF1 levels remain unchanged [1]. Why this occurs is not known, especially when in TAF4b-null mice liver regeneration and B-cell proliferation is not compromised.

How TAF4b induction is signaled is not fully understood. A very recent study reported that c-Myc induces TAF4b expression through a non-canonical E-box site [17]. c-Myc mRNA has been shown to be induced within the first 2 hours following hepatectomy but conditional deletions of c-Myc in the liver demonstrate that it is not required for regenerative proliferation following partial hepatectomy [18-20]. There are many redundant pathways in liver regeneration and it is possible that induction of c-Myc, though non-essential, leads to expression of numerous downstream targets.

Although TAF4b is present in a number of cell types, it is becoming clear that its essential function is in the testis and ovary. How and why a TFIID variant with such a specific reproductive function has evolved is still unknown but its presence is absolutely necessary in the gonad.

Materials and Methods

Surgery and liver collection

Hepatectomies were performed as described in Greene and Puder [6]. Briefly, mice were anesthetized using insoflurane prior to surgery. 50% hepatectomy consisted of ligature and removal of the left and left medial lobe through a mid-abdominal incision followed by suture of incision. Sham surgeries involved incision, manipulation of liver without removal followed by suture of incision. Mice recovered in cages on a 37° warm plate during the hours following surgery. Two-thirds hepatectomy was performed in rat as described by Sanders and Gruppuso [21]. A pregnant mouse (TAF4b het-het cross) was sacrificed and fetuses removed at embryonic day 17.5. Fetal liver was removed and all lobes were used in subsequent experiments. All liver samples were weighed upon retrieval.

Quantitative RT-PCR

Total RNA was isolated from liver samples using Trizol reagent. 1 μg RNA was used to make cDNA with iScript following the manufacturer's instruction (Bio-Rad). Real-time PCR reactions were performed in triplicate using 2 μl cDNA template, ABI Power SYBR green PCR master-mix and gene specific primers in the ABI 7500 Real Time PCR System. Dissociation curves were generated and PCR products were run on an agarose gel to verify amplification of a single product. qPCR data were analyzed by the ΔΔCt method. Relative expression levels were normalized to 18S ribosomal RNA levels to correct for equivalent total RNA levels.

Quantitative RT-PCR primers

	Forward (5'-3')	Reverse (5'-3')
TAF1	GAA AGG TGT TGC ACT TTT TAC GG	AGG AGC ATA GTC ATA GTT CCA CA
TAF3	TGA GAA CTT CTT GGG TAA GAG GC	TAG GGA GTC CCC TTT AGT GCT
TAF4	ATC TCC ACT GTG CAG GCTT CC	GGT CAG CTG CCG TGC AAT A
TAF4b	GAT GTT ACT AAA GGC AGC CAA GAG T	CTG CTC TGG ATC TTC TTT ATT GGA G
MED26	GAC TCC CAG AGC AAC ATC CG	CCC TAG TCG TGT CTC CTC CAG
Cyclin E1	AAT TGG GGC AAT AGA GAA GAG GT	TGG AGC TTA TAG ACT TCG CAC A
Cyclin D1	CCT CCC CC TTT TCT CTG C	CTG GAG GCT GCA GGA CTT T
Cyclin D2	GCT CTG TGC GCT ACC GAC TT	AAT CAT CGA CGG CGG GT
18S rRNA	CCG CGG TTC TAT TTT GTT GG	GGC GCT CCC TCT TAA TCA TG

Western blot analysis

Samples from rat liver nuclear extract were generously provided by Dr. Gruppuso's lab and prepared as described in Sanders and Gruppuso [21]. Following separation by SDS-PAGE and transfer to nitrocellulose, blot was cut and probed with antibodies against TAF4b [22] and β-tubulin (Neomarkers). Blot was re-blocked and re-probed with antibodies against TAF4 (BD Biosciences).

BrdU immunohistochemistry

47 hours following 50% hepatectomy, mice were injected with BrdU (Sigma) at 100 mg/kg body weight and sacrificed one hour later. Livers were fixed in formalin overnight, embedded in paraffin and sectioned at 7μm. Slides were deparaffinized in xylene and hydrated in graded alcohol. Antigen retrieval was performed in a steamer with sodium citrate buffer pH 6.0. Sections were washed with 3% hydrogen peroxide to quench endogenous peroxidase, blocked with normal goat serum/BSA/tween20, and probed with antibodies against BrdU (Sigma, B8434). Slides were incubated with horseradish peroxidase conjugated secondary antibodies, washed and allowed to react with 3-3′ diaminobenzidine and nickel (Vectashield DAB substrate kit, Vector Labs).

Slides were counterstained with Nuclear Fast Red and mounted with Cytoseal. Images were taken using the Aperio Scanscope CS. Five sections per slide with 200-300 nuclei each were scored for total nuclei and BrdU positive nuclei.

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I would like to thank Kimberly Seymour and Connie Lee for tirelessly performing all mouse hepatectomies and assisting with sample preparation. I would also like to thank Dr. Phil Gruppuso and Dr. Jill Sanders for reagents and helpful discussion.

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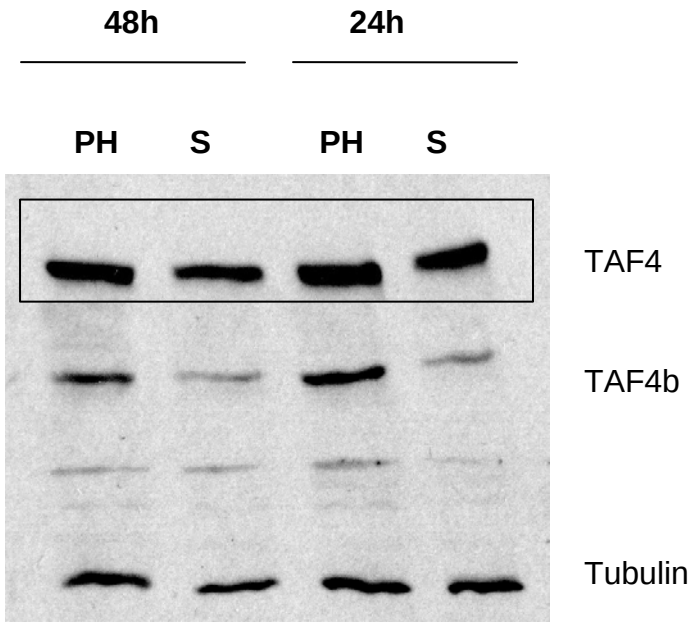


Figure 1. TAF4b protein induction in liver following hepatectomy. Nuclear protein extract was taken from rat liver following 2/3 removal or sham operation at 24 or 48 hours post-surgery. Western blot analysis was performed probing with antibodies against TAF4, TAF4b and β-tubulin.

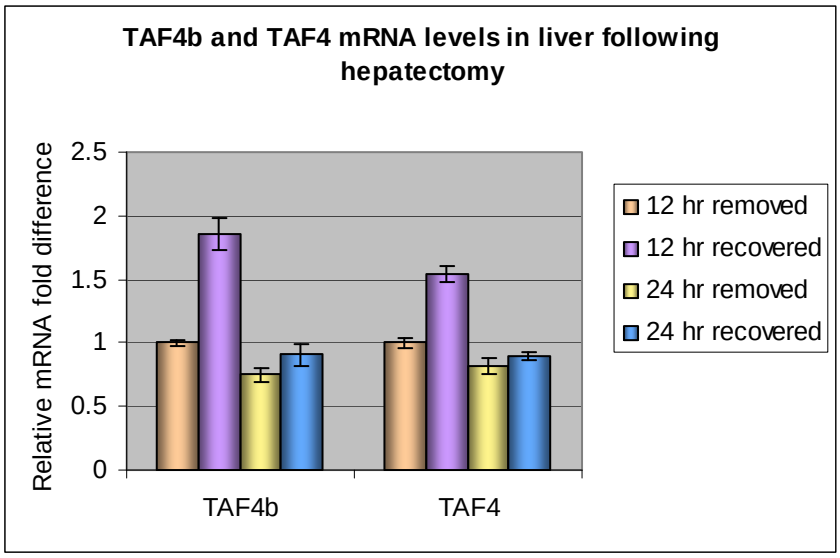


Figure 2. TAF4b and TAF4 mRNA levels in liver following hepatectomy. cDNA was made using total RNA isolated from the removed left lobe (0 hours) and the recovered right lobe at 12 or 24 hours post-hepatectomy. Gene expression of TAF4b and TAF4 was measured using qRT-PCR. Data was normalized to 18S rRNA. Error bars represent standard deviation.

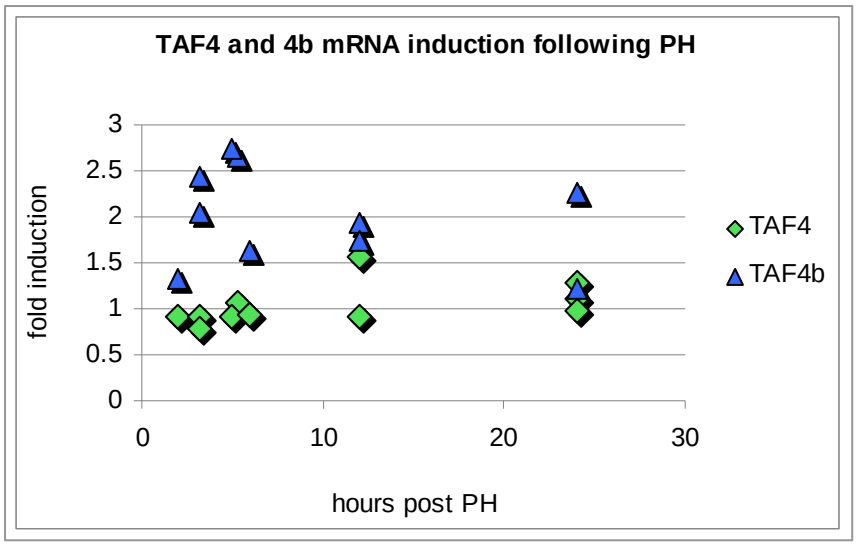


Figure 3. Induction of TAF4 and TAF4b following partial hepatectomy. Ratio of mRNA level between removed left lobe and recovered right lobe determined fold-induction for TAF4 and TAF4b at the indicated time points following hepatectomy. n=11.

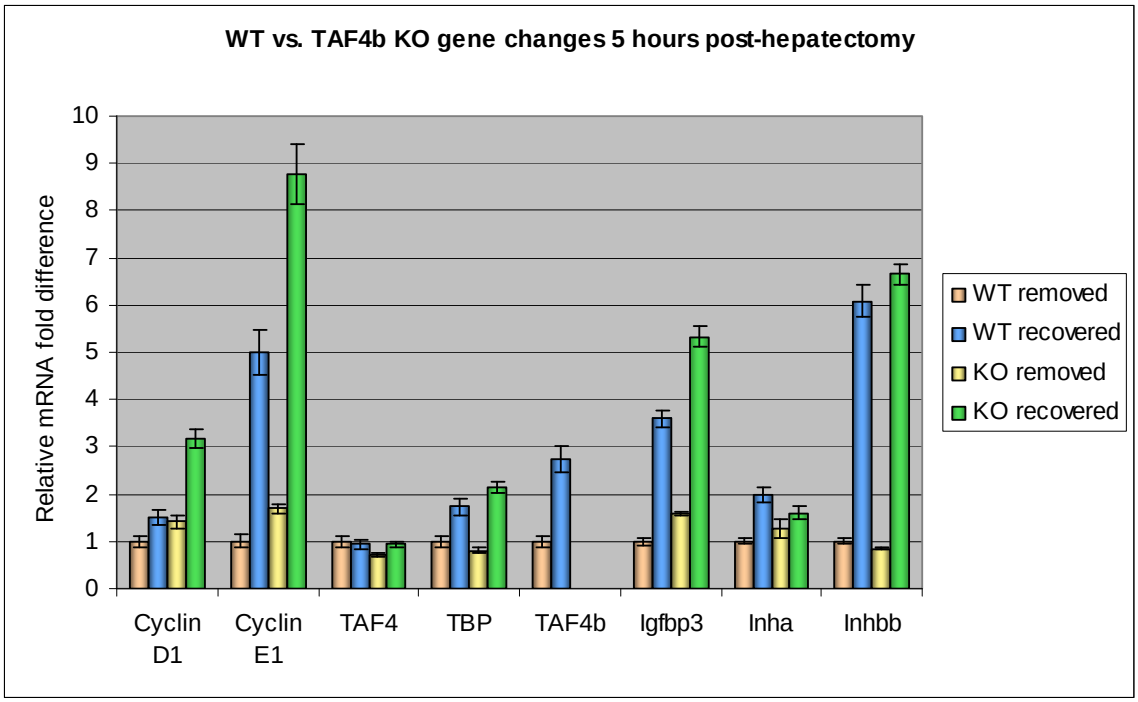


Figure 4. Gene expression changes following hepatectomy in TAF4b-null and wildtype liver. cDNA was made using total RNA isolated from wildtype and TAF4b-null mice livers. The removed, left lobe was harvested at 0 hours and recovered, right lobe was harvested at 5 hours following hepatectomy. Target gene expression was measured using qRT-PCR with primers specific to Cyclin D1, Cyclin E1, TAF4, TBP, TAF4b, Igfbp-3, Inha and Inhbb. Data was normalized to 18S rRNA. Error bars represent standard deviation.

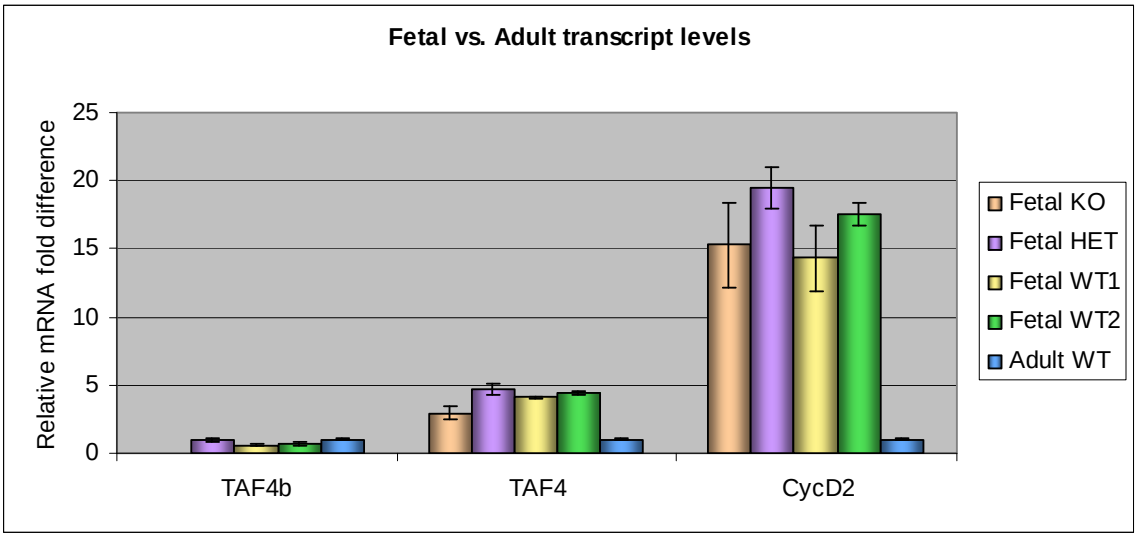


Figure 5. Fetal vs. adult transcript levels across TAF4b geneotypes. cDNA was made using total RNA isolated from adult liver and embryonic day 17.5 liver of TAF4b-null, heterozygous and wildtype littermates. Gene expression of TAF4b, TAF4 and Cyclin D2 was measured using qRT-PCR. Data was normalized to 18S rRNA. Error bars represent standard deviation.

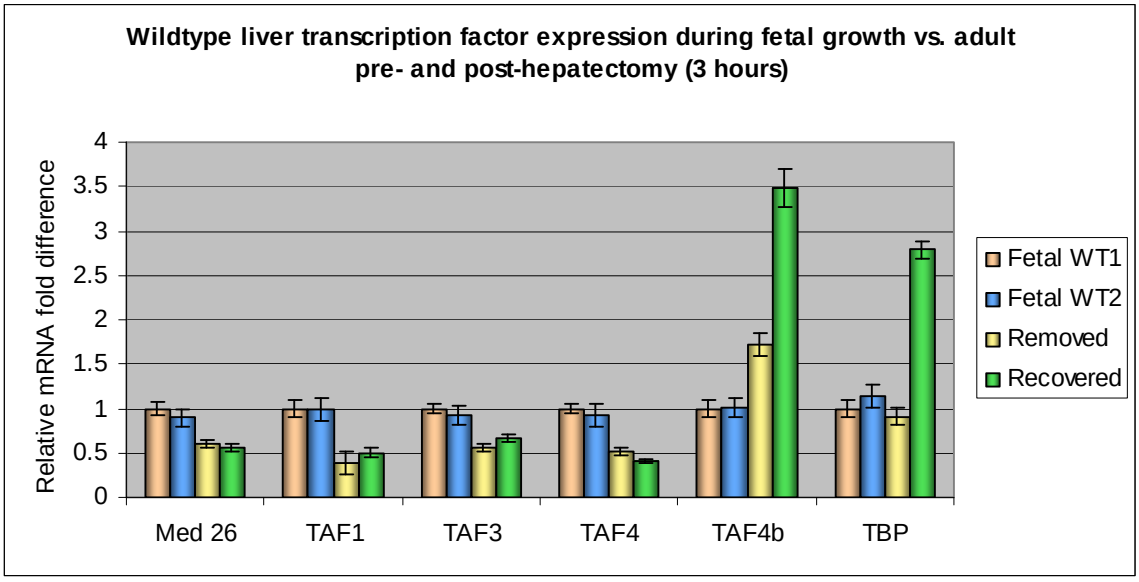


Figure 6. Wildtype liver transcription factor expression during fetal growth vs. adult pre- and post-hepatectomy. cDNA was made using total RNA isolated from embryonic day 17.5 liver, adult removed left lobe (0 hours) and the recovered right lobe at 3 hours post-hepatectomy. Target gene expression was measured using qRT-PCR with primers specific to Med 26 (Mediator 26), TAF1, TAF3, TAF4, TAF4b and TBP. Data was normalized to 18S rRNA. Error bars represent standard deviation.

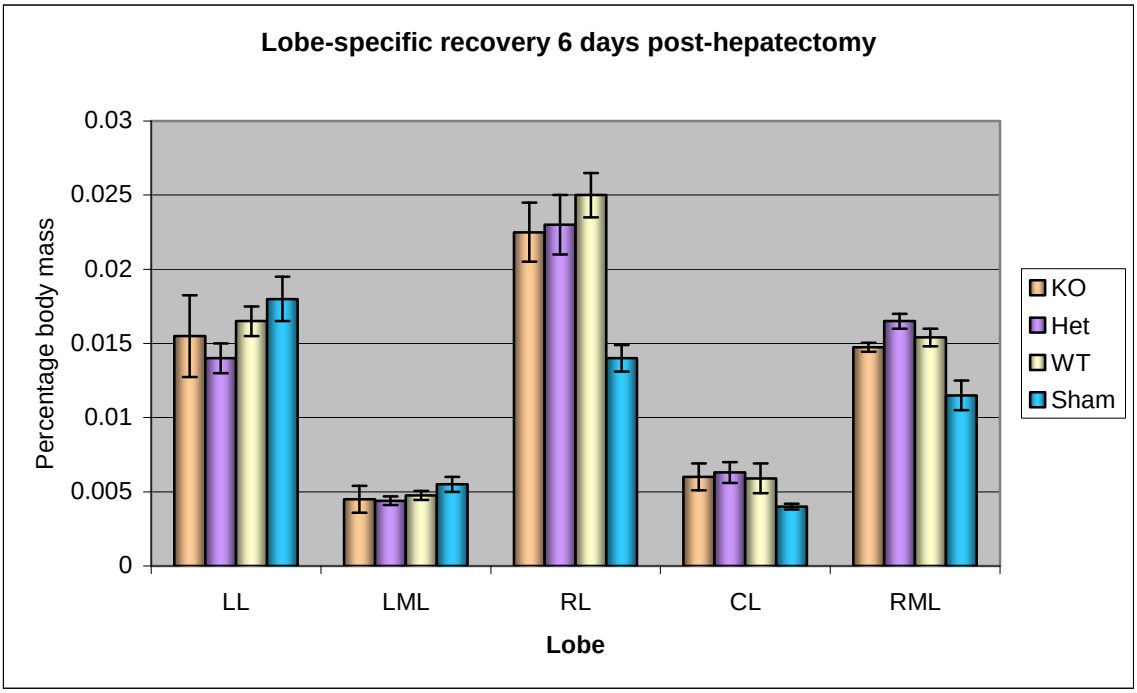
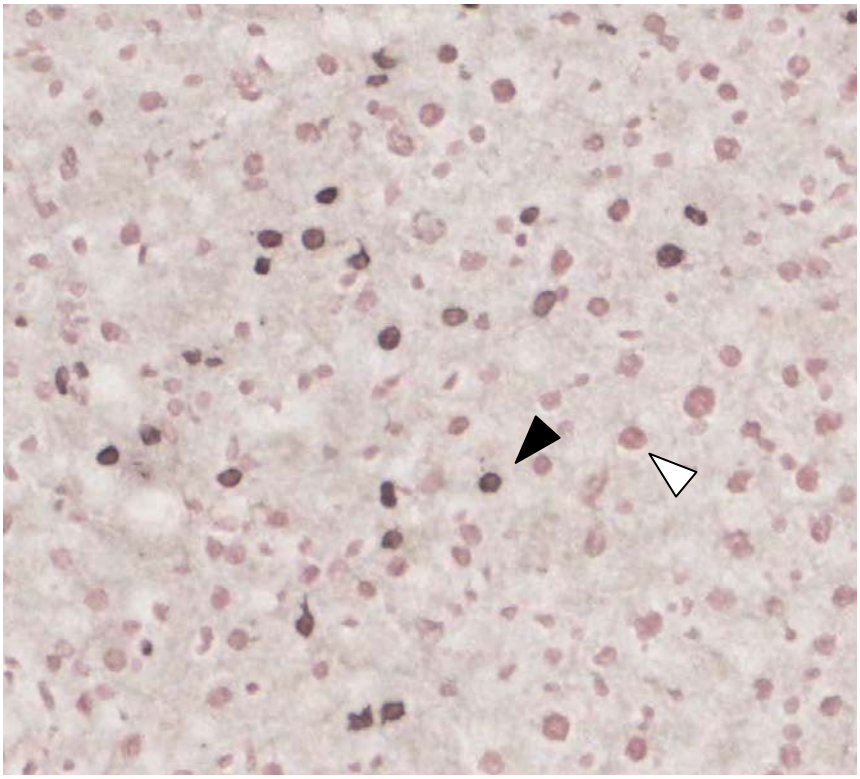


Figure 7. Mass recovery across TAF4b genotypes 6 days post-hepatectomy. Mass is represented by percentage of body weight. Left lobes were removed during hepatectomy with the exception of the sham, which was collected at 6 days. LL, left lobe; LML, left medial lobe. Right and caudate lobes were collected after 6 days of re-growth. RL, right lobe; CL, caudate lobe; RML, right medial lobe. TAF4b KO n=3, TAF4b Het n=3, WT n=2, Sham n=3. Error bars represent standard deviation.

A



B

	TAF4b-null	WT
Total nuclei counted	1280	1468
BrdU positive nuclei	158	180
% BrdU positive nuclei	12.3%	12.2%

Figure 8. Immunohistochemical detection of BrdU at 48 hours post-hepatectomy. **(**A) Representative sample of histological detection of BrdU. Black arrowhead indicates BrdU positive nucleus, white arrowhead indicates BrdU negative nucleus. (B) Scoring of TAF4b-null and wildtype livers following hepatectomy.

Chapter 5

Differential expression of RNA Polymerase II transcriptional machinery in regulation of cellular identity

Abstract

Establishment and maintenance of unique gene expression programs is required for cellular identity. Although the majority of proteins involved in RNA Polymerase II (Pol II) transcription are conserved from yeast to human, it is becoming clear that these general transcription factors (GTFs) are important in metazoan differentiation. Composition of large, multi-subunit complexes such as Mediator and TFIID are dynamic. Even slight changes in subunit composition can influence RNA Pol II promoter recognition and transcriptional output. Through evolution, variant components of the general transcriptional machinery have arisen, adding another layer of complexity to regulation of metazoan gene expression. One such variant, TAF4b, has been identified in mammals and has very specific roles in regulating both male and female fertility. When TAF4b was identified, it was believed to be a gonadal-enriched paralog of the broadly expressed TAF4. Here we show that contrary to popular belief, general transcription factors display varying degrees of tissue specificity. Recent findings reveal that undifferentiated cells express high levels of transcription factors. We demonstrate the reduction of GTF components through differentiation of J1 embryonic stem cells and propose that in addition to GTF variants, the regulation of original TFIID and Mediator subunits are instrumental in establishing and maintaining gene expression, conferring tissue identity.

Introduction

As our understanding of the large, multi-subunit complexes involved in RNA Pol II transcription in mammals increases, it is becoming evident that they are not just general co-factors but are involved in creating specialized gene expression programs. TFIID and the Mediator complex are of particular interest due to the variability that can exist in their subunit composition. Gene-deletion studies in mouse have demonstrated that disruption of individual subunits results in specific developmental defects, the majority of which are embryonic-lethal. The variety of differentiation processes impacted by the loss of TFIID and Mediator subunits suggests that these global transcription factors are involved in numerous specialized differentiation events.

The Mediator (MED) complex was first identified in *S. cerevisiae* as a large, multi-subunit complex involved in Pol II transcription [1]. With over 20 proteins, and multiple enzymatic activities, Mediator serves to bridge interactions between transcription factors, components of RNA Pol II and GTFs [2]. Without known, intrinsic, DNA binding capabilities, Mediator uses protein-protein associations to exert control over both basal and activated transcription, functioning as both a co-activator and co-repressor [3, 4]. The composition of Mediator is dynamic and numerous variations of the complex have been isolated. In mammals, approximately 30 subunits have been isolated. While not every subunit is present in every isolated complex, the most complete form of Mediator is well established and is composed of 3 main regions- the core, tail and CDK module [5] (Figure 1). The central core of tightly associated proteins is composed of MED4, MED6, MED7, MED10, MED11, MED14, MED15, MED17, MED18, MED20, MED21, MED26, MED30 and MED31. The tail region of Mediator is composed of

MED16 MED23, MED24 and MED 25 and, generally, these subunits are more weakly associated. An even more transiently associated region is the CDK module which contains MED12, MED13, Cdk8 and cyclin C.

Mediator functions both as a co-activator and co-repressor, governed by its subunit composition at a given promoter. It is generally believed that Mediator containing the CDK module represents a repressive complex, while inclusion of MED26 represents an activating complex, and that CDK and MED26 are mutually exclusive within Mediator [6]. Consistent with this hypothesis is that co-purification of Mediator complexes using FLAG-tagged MED26 resulted in isolation of more complexes associated with the RNA Pol II and less with the CDK module compared to pull-down assays using other FLAG-tagged Mediator subunits. It was also shown that complexes associated with Pol II are more transcriptionally active [7]. Although the Mediator core is usually purified containing all the subunits, a variant complex without MED26 and MED1 has been purified and other isolations of Mediator contain sub-stoichiometric levels of MED26 [8-10]. The most well defined Mediator-related complexes are PC2, ARC, TRAP and CRSP, which although heterogeneous in nature, are composed of Mediator subunits and function in transcription. It is possible that the subtle differences in these Mediator and Mediator-like complexes confer specificity, influencing target gene transcription. Subunit variations likely alter the activity of Mediator according to the cell's transcriptional requirement. Regulation of transcription by Mediator is not as simple as switching groups of subunits, however. Order of contact with other components involved in Pol II transcription induces conformational changes which lead to increased or decreased affinity for other binding partners. The number of possible associations

between several large, multi-subunit complexes affords mammalian transcriptional gene regulation an enormous opportunity for specificity.

In mammalian cells, CDK containing Mediator has been shown to negatively regulate transcription through phosphorylation of TFIIH. Mediator CDK8 phosphorylates the cyclin H component of TFIIH to prevent Pol II CTD phosphorylation and transcription activation [11]. Two functional complexes comprise TFIIH; the cyclin-activating kinase complex (CAK) and a core complex. The core complex consists of XPB and XPD helicases, p62, p52, p44 and p34 and contains bi-directional DNA helicase activity [12, 13]. The CAK is composed of CDK7(p40), cyclin H(p38) and MAT1(p32) and is responsible for phosphorylation of Pol II CTD at serine 5 [14]. Possessing ATPase, helicase and kinase activity, TFIIH is required for transcription initiation and promoter clearance. TFIIH's kinase activity is not limited to Pol II and can also phosphorylate numerous transcriptional activators such as p53, RARα, RARγ, and ERα [15-18].

Although homologs of CDK8/Cyclin C and Cyclin H exist in *S. cerevisiae*, the particular negative regulation reported in mammalian cells is not observed in yeast and may represent a mechanism that has evolved to regulate the metazoan genome[19]. One process which must have a much higher level of complexity between yeast and metazoans is cellular differentiation. Although some Mediator subunits are required for cell viability in both yeast and metazoans, disruptions of other subunits have specific, metazoan effects. Mediator subunits 6, 17 and 21 are essential for both yeast cell viability and mouse embryo progression through the blastocyst stage [20]. MED1-null mice die with heart and neural defects by embryonic day 8.5 (E8.5) [21]. MED24-null mice have

a broader window of embryonic death occurring between 8.5 and 11.5 days, resulting from pleiotropic defects in activator-dependent transcription [22]. Although not lethal, mutations in MED 10 and 13 in metazoans also result in specific, developmental defects [23].

In addition to Mediator and TFIID, other GTFs are involved in metazoan-specific differentiation events that are not required in lower eukaryotes. TFIIS, for example, is an elongation factor that is non-essential in yeast, but when inactivated in mouse, results in embryonic lethality due to a defect in erythroblast differentiation in the fetal liver [24]. Recent work by Helenius *et al.* demonstrated that the CDK7 and p32/MAT1 components of TFIIH are undetectable in adipose tissue despite the view that TFIIH factors are ubiquitously expressed, and that CDK7 prevents adipogenesis by inhibiting PPARγ [25]. TFIIE is composed of α and β subunits that act as a heterodimer to work with TFIIH in promoter melting and clearance [2]. An interesting role for this general transcription factor was elucidated when it was found to interact with a specific class of homeodomain factors in *Drosophila* [26]. Antennapedia and Abdominal-B, but not Even-skipped, bind to the β subunit of TFIIE, and this interaction stimulates transcription. Prior to this experiment it had been shown that another homeodomain containing protein, mouse Msx-1, interacted specifically with TBP but not TFIIE, and in that case, the interaction lead to transcriptional repression [27]. Together, these studies have challenged the current, and somewhat limited, view of basal transcription factors in mammalian tissues. It is apparent that GTFs are critical players in specifying cell-lineage in metazoans.

It is well known that specialized transcription factors such as Oct4, Sox2, Klf4 and Nanog play an important role in keeping cells in a pluripotent state while others, such

as MyoD, are required to initiate cell-type specific transcription, but it is becoming clear that GTFs and Mediator are involved in differentiation as well. Here, we use ES cell differentiation as well as examination of several differentiated mouse tissues to observe the varied expression levels of GTFs and Mediator components between mammalian cell types.

Results and Discussion

Mediator subunits are reduced to varying degrees upon embryonic stem cell differentiation

J1 embryonic stem (ES) cells can be induced with retinoic acid (RA) to differentiate into neuronal precursor cells that stop dividing upon terminal differentiation. J1ES cells were exposed to RA over a 16 day period and differentiation was deemed nearly complete by lack of expression of pluripotency markers such as Sox2 and Oct4 (data not shown). Total protein extracts from pre- and post- differentiated J1 ES cells were assayed by Western blot for levels of several factors involved in transcription (Figure 2). Testis extract was used as a control for expression in a tissue that has both terminally differentiated cells and proliferating cells. Mediator components MED1, MED6, MED12, MED18 and MED26 were found to have varying degrees of differential expression between proliferating and differentiated cells. MED6 and MED18 are components of the core complex and their levels remain unchanged with differentiation. MED1 levels are slightly reduced. MED12 and MED26 levels are markedly decreased following differentiation.

MED12 is a component of the CDK module of Mediator and its association with the core complex usually indicates a transcriptionally repressive state. Both MED12 and MED26 have been shown to be involved with silencing of neuronal genes in non-neuronal tissue and preventing premature expression in neural progenitor cells with the transcription factor REST [28]. Reduction, but not complete elimination of MED12 and MED26 may suggest that a lower level of these proteins is needed to prevent ectopic silencing of neuronal genes after differentiation, but there may be processes independent of REST-Mediated silencing for which they are still required, explaining why they are not completely eliminated. From a proliferation standpoint, lowering the level of MED12 would decrease the amount of CDK complex available, and therefore could allow for increased association of Mediator with Pol II, favoring a transcriptionally active state. Reduction of MED26, however, could lead to the opposite effect and reduce the interaction of Mediator with Pol II, resulting in a less transcriptionally active complex. Although total protein levels of MED12 and MED26 in the differentiated cells is reduced, the current study does not give any information as to the assembly of Mediator, making it impossible to gauge the effects of the lowered protein levels. Alternately, as opposed to previous studies using *in vitro* reconstitution systems to determine transcriptional activity of Mediator/Pol II complexes, additional interacting factors *in vivo* may lead to different outcomes. For example, inclusion of MED26 into Mediator could lead to a complex with increased ability to bind Pol II, which in *in vitro* systems causes increased transcriptional output, but *in vivo*, perhaps there are cell- or promoter-specific scenarios where the presence of additional factors leads to repression by a MED26-containing Mediator.

HP1α is induced upon embryonic stem cell differentiation

Heterochromatin protein 1 (HP1) is an important factor in negative gene regulation. Recruited by tri-methylated histone 3 lysine 9, HP1 binds to DNA, leading to chromatin condensation and gene silencing. HP1 seems to prevent pre-initiation complex assembly by preventing components of Mediator or TFIID from binding to the promoter [29]. Interestingly, we do not detect HP1 in proliferating J1ES cells, but after differentiation and in testis extract, HP1 can be readily detected. The lack of HP1α agrees with previous data documenting the transition of chromatin conformation during differentiation [30] and the finding that ES cells are in a state of hyper-transcription with an open chromatin conformation [31, 32]. Heterchromatin does exist in ES cells in the form of chromocenters, but these cells do not have the hyper-condensed regions observed in differentiated cells [31]. Efroni *et al.* found that global levels of transcription were higher in R1 ES cells before differentiation and that expression included repetitive sequences, mobile elements, and coding genes both housekeeping and cell-type specific in nature. It was also reported that ES cells expressed very high levels of chromatin remodeling factors and general transcription machinery. A model of differentiation has been established whereby ES cells maintain an open chromatin conformation that allows for hypertranscription, carried out by GTFs. Upon receiving differentiation signals, the genome is restricted through specific chromatin compaction events leading to lineage specification.

TFID subunits are differentially reduced or eliminated upon embryonic stem cell differentiation

Upon differentiation, we saw a reduction of several TFIID components. While all were abundant in ES cells, after differentiation TAF4b became nearly undetectable and while TAF4, TAF1 and TBP were still present after differentiation, they were dramatically reduced. We also observed a decrease in the largest Pol II subunit, Rpb1. Together these changes in protein levels agree with the idea that a higher level of transcription in ES cells requires more general transcription machinery.

Loss of nearly every TAF in yeast is lethal, with deletion of $yTAF_{II}145$ (TAF1) TSM1 (TAF2), and $yTAF_{II}90$ (TAF5) resulting in cell cycle disruption [33-35]. Attempts to knock-out TAFs in mouse lead to embryonic lethality occurring at various time points during gestation, indicating the knockout cells are initially viable but cannot overcome deficiencies at later developmental time points. TAF4-null mice show embryonic lethality after day E9.5, indicating that this factor is not essential for cell viability, but for later processes in development [36]. TAF8 knockout mice have earlier defects and die by day E4.0 with the trophoblast intact and defects in the inner cell mass [37]. Trophoblasts provide the embryonic contribution to the placenta while the inner cell mass will undergo many more differentiation steps to form the fetus [38]. TAF10 knockout mice also have early defects and die at E5.5 from defects in inner cell mass cells. Trophoblast cells isolated from TAF10 -/- blastocyts were viable, indicating that TAF10 is not required for transcription in all cells, but is necessary for cell-type specific transcriptional events in early embryogenesis [39]. When TAF10 is targeted for deletion in F9 embryonic carcinoma cells, under normal conditions, the deletion is lethal. However, if the cells are

differentiated with RA to endodermal cells, they survive. This finding highlights the temporal requirement for a specific TAF and its dispensability at a different developmental stage. It is clear that individual TAFs act at different time points in embryonic development, but survival of trophoblast cells indicates that they are not absolutely required for cell viability.

Differentiation of myoblasts to myotubes is highly dependent on TFIID switching from the canonical form to a much more limited complex that contains only TAF3 and the TBP-related factor, TRF3 [40]. Upon differentiation, there is a down regulation of TAF1, TAF4 and TBP, while levels of TAF3, TRF3 and the other GTFS (TFIIA, B, E, F and H) remain constant. This finding not only implicates the general transcriptional machinery in cell-lineage specification, but also demonstrates that removal of currently existing proteins is a crucial step in the differentiation process.

HP1 can affect chromatin's association with transcriptional machinery in order to induce gene silencing. For some target proteins, HP1 is only able to prevent their binding to chromatin, while for others, HP1 is able to actively displace them from chromatin. Components of Mediator, the GTFs and RNA Pol II show differential responses to the presence of HP1 at promoters. HP1 prevents assembly of the majority of proteins tested (Pol II, MED1, CDK8, TFIIH, TFIIE, TFIIB, TAF1, TAF4, and TBP), while it only actively displaces a few (Pol II, MED1, CDK8, TAF1, TAF4, and to a lesser degree, TBP). MED23, MED25 and MED6 appear to be resistant to disassembly, and MED6 is only marginally blocked from assembly by the presence of HP1 [29]. It is interesting to note that we found a correlation between proteins that decreased with RA differentiation and the transcription factors that are able to be actively displaced by HP1, but very little

or no change in the factors that are resistant to disassembly by HP1. Factors that are blocked from assembly by HP1 are not decreased during the differentiation program used in this study.

Differentiation to different cell types likely results in down-regulation of different transcription factors in order to specify appropriate gene-expression. Perhaps inactivation of certain GTFs and Mediator components is essential to shut off pre-existing transcription. This could explain why HP1, one of the first proteins to act in the transition from euchromatin to heterochromatin, can remove transcription factors from chromatin. An interpretation of the results observed during the differentiation in this experiment, combined with HP1 displacement assays, is that during differentiation a number of transcription factors need to be removed in order to establish lineage-specific gene expression, and for their efficient removal, HP1 is required to dissociate them from chromatin.

GTF and Mediator components are differentially expressed in mouse tissues

If GTFs and Mediator components play such an important role in cell type-specific gene expression, analysis of their presence in different tissues would be informative. To address this, we performed western blots on several different, adult mouse tissues to determine the expression pattern of GTFs and Mediator components (Figure 3). RNase protection assays have reported that TAF4 is expressed at similar levels in a variety of tissues, while TAF4b is highly expressed in the gonads and spleen [41]. While TAF4b protein expression is consistent with its mRNA expression, TAF4 protein levels are not. Like TAF4b, TAF4 protein levels are highest in the gonads and spleen, and TAF4b and TAF4 protein expression patterns appear almost identical. Interestingly, even Pol II

expression varies by tissue type. Like TAF4 and TAF4b, the highest expression appears to be in the gonads and spleen, although strict interpretation of this blot is not possible due to the multiple bands near the expected molecular weight of Rpb1. The XPB/p89 subunit of TFIIH is also expressed as multiple molecular weight products, and its expression also varies by tissue. TFIIB has a distinct expression pattern, with its highest level in the brain. Of the Mediator complex components tested, MED26 and MED12 show quite varied expression among tissues, while MED18 is more evenly expressed. Generally, we observed higher levels of the tested factors in the testis, ovary and spleen, while the kidney and heart had the lowest levels. Although these trends exist, each factor seems to have its own distinct expression pattern, likely contributing to specific gene expression profiles in that tissue.

Methods

Embryonic stem cell differentiation

As described in Tantin *et al* [42], differentiation of J1 ES cells occurred in the presence of 10^{-7} M (or 100 nM) retinoic acid (RA) over a 16 day period. ES cells were cultured in DMEM + HEPES supplemented with 1 mM glutamine, 1 mM sodium pyruvate, and 1 mM MEM nonessential amino acids (Invitrogen) plus 15% ES cell-qualified heat-inactivated fetal bovine serum (HyClone), 50 μM 2-mercaptoethanol (Sigma), and leukemia inhibitory factor (LIF/ESGRO, Chemicon).

Western blot analysis

Whole adult mouse tissues were Dounce homogenized in extraction buffer (0.2 M KCl, 0.1 M Tris, 0.2 mM EDTA, 0.1% NP-40, 10% glycerol, 1mM PMSF), incubated on ice

for 50 minutes and centrifuged for 10 minutes to pellet cellular debris. Soluble protein was mixed with Lammeli buffer. J1 ES protein extracts pre- and post-differentiation were generously provided by Dr. Fairbrother's lab and prepared as described in Tantin *et al* [42] . Protein concentration was determined using the Bradford assay, and equivalent amounts of total protein were loaded per well. Following protein separation by SDS-PAGE and transfer to nitrocellulose, blot was cut and probed with antibodies against TAF4b [43], β-tubulin (Neomarkers), TAF1 (Santa-Cruz), TBP (Abcam), TAF4 (BD biosciences) and TFIIH (Santa Cruz). The following antibodies were generously provided by Dr. Marr of Brandeis University: HP1α, TFIIEβ, Med1, Cdk8, Cyclin H, MED6, MED12, MED18, MED26, Pol II-Rbp1, TFIIS, and TFIIB.

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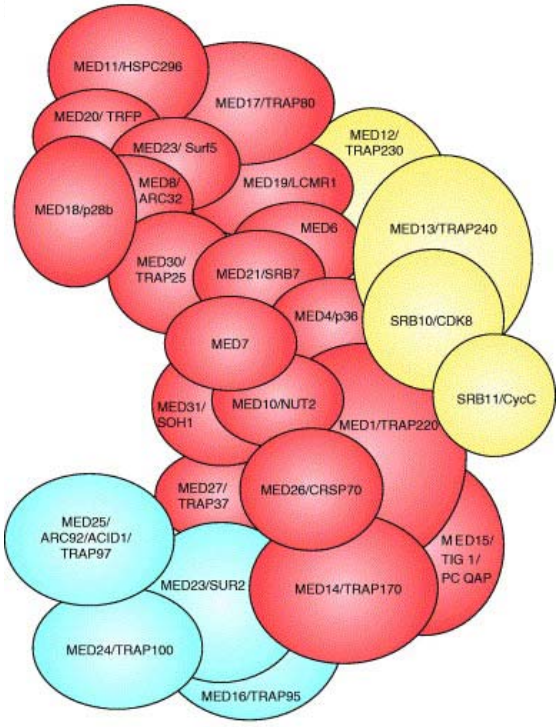

Figure 1. Human Mediator Complex. CDK region is in yellow, core region is in red and tail region is in blue. Adapted from *MEDIATOR* [5].

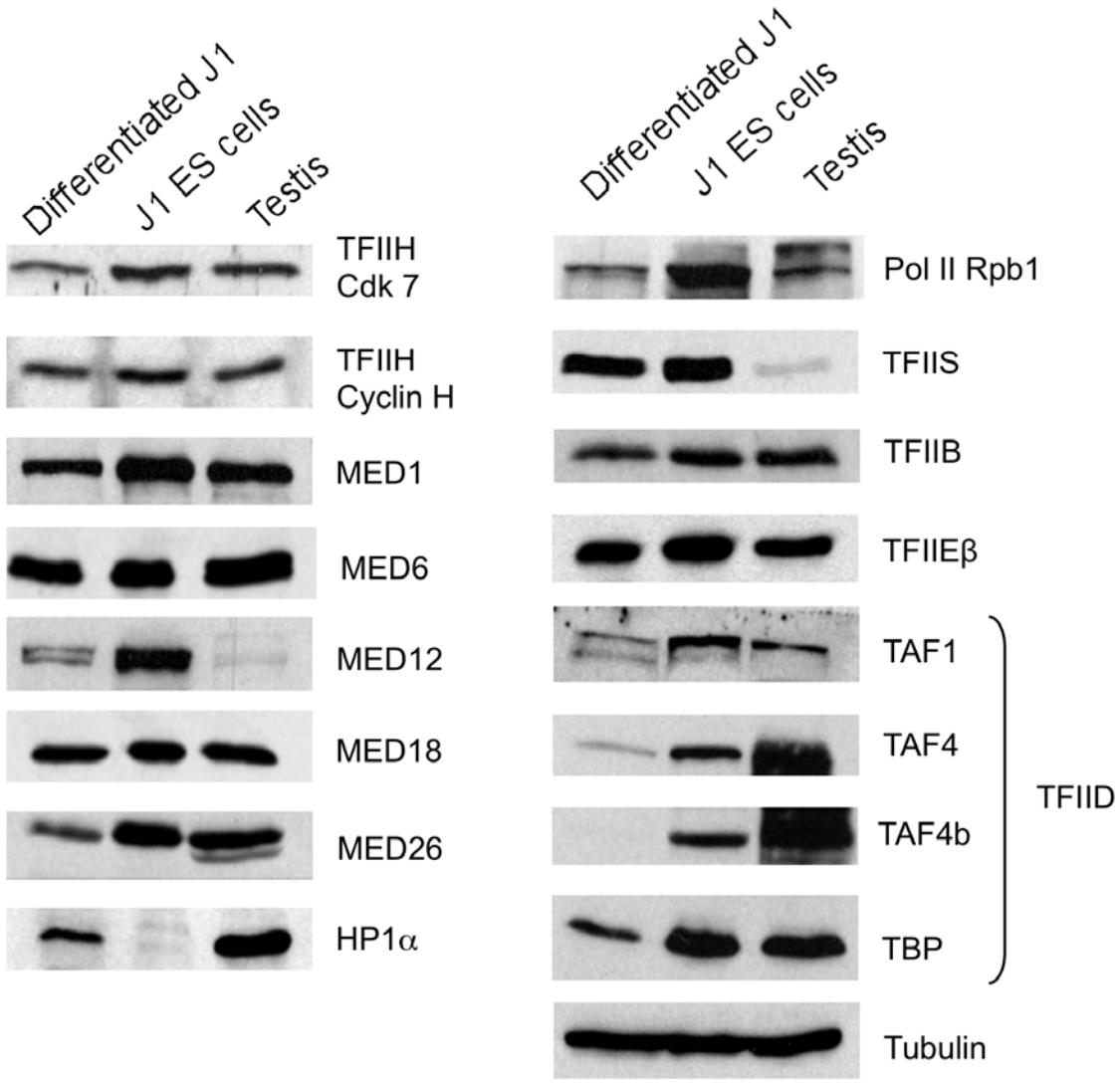

Figure 2. Dynamics of general transcription factor subunits upon J1 ES cell differentiation. Protein extracts from J1 ES cells pre- and post-differentiation and adult mouse testis were subject to Western blot analysis using indicated antibodies. Tubulin serves as a loading control.

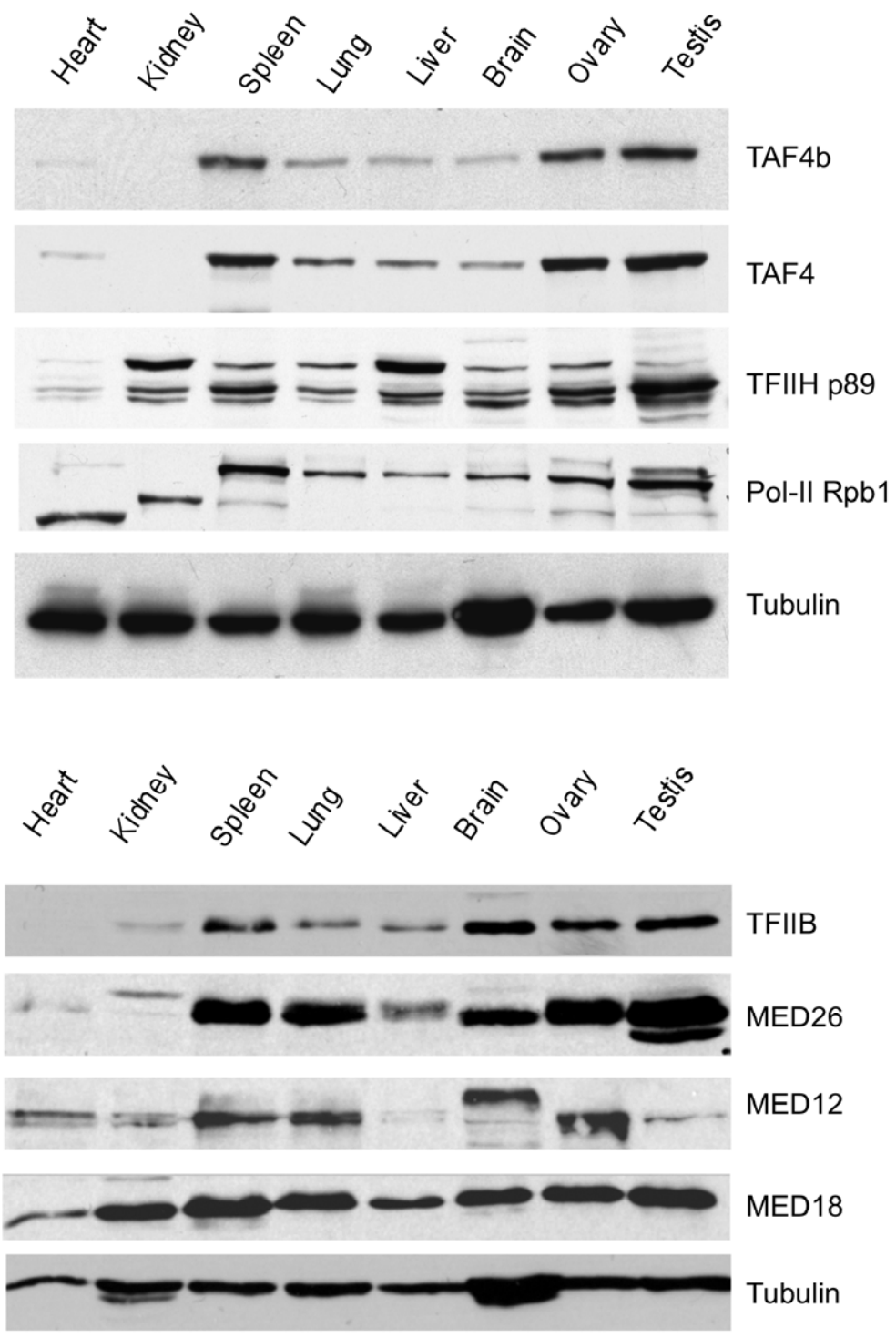

Figure 3. Differential expression of Pol II transcription machinery between tissues. Western blot of mouse tissue extracts probed with indicated antibodies. Tubulin serves as a loading control.

Chapter 6

Discussion and future directions

Genome diversification through transcription factor evolution

These studies have highlighted the specific requirement for TAF4b in mammalian fertility. Although TAF4b is expressed in multiple organ systems, its presence appears non-essential for function or development outside of the gonad. The data presented here give insight into how a specialized mode of evolution has contributed to diversification of metazoan genome expression.

TAF4 is a critical component of TFIID and is partially conserved from yeast to man. Loss of the TAF4 yeast ortholog, yTAF48, leads to cell death in a cell cycle stage-independent manner [1]. Metazoans differ from yeast in that the composition of TAF4 lacks an N-terminal co-activation domain, and no TAF4 variant exists within the yeast genome. These are very important differences when assessing the roles of paralogous TAF4 and TAF4b in mammals. The co-activator domain that is present in TAF4/4b of multi-cellular organisms has allowed for the increased diversity of transcriptional regulation required for tissue specific gene expression programs [2].

The emergence of TAF4b occurred from a yet to be characterized TAF4 gene duplication event. Gene duplications are evolutionarily advantageous because the functional redundancy of the proteins protects from negative effects that may arise from spontaneous mutation of the original gene [3]. Because TAF4 is an essential gene, its duplication is beneficial to survival. Along with protecting from deleterious mutations, gene duplication is a powerful mechanism to evolve transcription factors with novel activities. Although the majority of the phenotypic variations between related species occur from changes in *cis* regulatory elements, and not the coding region of genes, there have been mutations that affect protein-protein interactions of a transcription factor [3].

While evolution requires mutations to occur, advantageous mutations in one cell type could be detrimental in another. By employing such mechanisms as tissue specific expression and alternative splicing, the pleiotropic effects associated with transcription factor mutations are reduced, allowing for mutation-driven transcription factor evolution.

If a transcription factor is broadly expressed, a mutation in the coding region that changes its function and is advantageous in one tissue or cell type is likely to be deleterious in other cell types. A large proportion of transcription factors are expressed in a tissue-specific manner. A comprehensive study of transcription factor expression found that of the estimated 1962 transcription factors, representing 8% of protein encoding genes, 605, or 30%, are expressed in a tissue-specific manner [4]. This implicates a large number of transcription factors in directing tissue-specific gene expression and allows for the selection of novel function without compromising transcription in other tissues.

Tissue-specific transcription factor expression can influence the properties of more broadly expressed transcription factors, thereby allowing new gene expression programs. A mutation affecting interaction only with a single, ubiquitous binding partner can cause specific changes in a certain tissue type without altering its function elsewhere. Tissue specificity provides a mechanism to allow for new regulatory functions to adapt while minimizing the risk of negative pleiotropic effects.

TAF4b has arisen from a gene duplication event, and since then has acquired new tissue specific activities that no longer make TAF4 and TAF4b functionally equivalent. There is much evidence that supports the idea that TAF4 and TAF4b can compensate for each other, but in the context of the development of an organism, these transcription factors have non-redundant roles [5-12]. We know from studies using *Drosophila* S2

cells that at a molecular level, TFIID assembly can be completed using only TAF4 or only TAF4b [13]. In the mouse, targeted deletion of TAF4 results in embryonic lethality by day 9.5, suggesting that TAF4b can compensate for TAF4 loss *in vivo* up to a certain developmental timepoint [10]. The resulting embryonic death could either be due to TAF4b becoming more restricted in its expression, or not being able to activate gene expression programs equivalent to TAF4.

Deletion of TAF4 in MEFs results in altered morphology and loss of serum-dependent growth regulation. Profiling of MEFs with and without TAF4 revealed that loss of TAF4 causes a number of genes to be up- or down-regulated. Gene expression changes in TAF4-/- cells could have been due to loss of TAF4- or gain of TAF4b-containing TFIID complexes. To determine which scenario occurred, Mengus *et al.* over-expressed TAF4b in MEFs with one copy of TAF4. The gene changes associated with overexpression of TAF4b mimicked loss of TAF4, leading the authors determine that TAF4b induces growth factor expression. The authors also attributed growth factor expression specifically to the activation properties of TAF4b because expression of a truncated TAF4, containing only the histone fold domain, returned the TAF4-/- MEFs to normal growth. Overall, the genes regulating cell proliferation are influenced by the competitive equilibrium between TAF4 and TAF4b-containing TFIID complexes [10].

While cell viability only requires either TAF4 or TAF4b, it is clear that these factors possess independent functions, both of which are required for different biological processes. In *Drosophila* and mouse, loss of multiple TAF paralogs each lead to fertility defects without affecting general development [14, 15]. Germ and somatic cell proliferation is essential to fertility. Due to the results demonstrating TAF4b's role in

promoting proliferation in MEFs and the disappearance of TAF4b with ES cell differentiation, we postulated that loss of TAF4b impairs proliferation leading to infertility in mice.

TAF4b-null mice exhibit gonadal-specific cell proliferation impairment

To address this question we assessed the ability of TAF4b-null ovaries to proliferate in response to administration of exogenous PMSG and estrogen. In wildtype mice, the granulosa cells of growing follicles exhibit a high degree of proliferation following hormone administration. As determined using BrdU incorporation, TAF4b-null ovaries have impaired proliferation in response to both PMSG and estrogen. Because proliferation was reduced in both treatments, it suggests that TAF4b-deficiency either compromises a shared component of those specific proliferation pathways, or there is a defect in general proliferation [8].

To determine if this proliferation defect was confined to the ovary we moved to a different model of *in vivo* cell division- post-hepatectomized liver. Preliminary data showed specific induction of TAF4b, but not TAF4 following hepatectomy in rats. We observed a similar response in mice with induction of TAF4b mRNA, peaking at about 5 hours following hepatectomy, while TAF4 levels remained fairly equivalent between pre- and post- operative livers. Although TAF4b is induced, TAF4b-null mice are not impaired in liver regeneration, indicating that loss of TAF4b does not inhibit proliferation following hepatectomy. It does not appear that TAF4 is compensating in TAF4b-null liver proliferation because TAF4 is similarly un-induced in TAF4b-null livers. The induction of TAF4b following hepatectomy is puzzling given that is does not seem to be required for regeneration. At a molecular level, the expression of several putative

TAF4b-target genes are induced in the liver following hepatectomy in both wildtype and TAF4b-null livers. Igfbp-3, inhibin α and inhibin βB have all been identified as TAF4b-target genes, but their induction levels following hepatectomy are equivalent between TAF4b-null and wildtype mice. This finding suggests that Igfbp3, inhibin α, inhibin βB and possibly other TAF4b-target gene expression is only conditionally dependent on TAF4b, perhaps in a cell-type specific manner.

TAF4b-target gene expression

Examining TAF4b-dependent transcription in granulosa cells yields conflicting results between *in vivo* and *in vitro* systems. While it had been reported that TAF4b over expression in culture induces the expression of granulosa cell specific transcripts such as inhibin α, inhbin βB, inhbin βA and cyclin D2, the levels of these transcripts do not differ in the ovaries of juvenile TAF4b-null mice. Using siRNA knockdown of TAF4b in the NT-1 granulosa cell line, we find decreased expression of c-Jun, cyclin D2, inhbin βA and Igfbp3. Together, these data suggest that the *in vivo* and *in vitro* situations are very different. Acute loss of TAF4b after siRNA-mediated knockdown results in specific downregulation of target genes. Perhaps in the TAF4b-null ovary, a compensatory mechanism has been put in place during development to correct gene expression defects in granulosa cells.

Interestingly, knockdown of TAF4 also reduces c-Jun, Igfbp3, and cyclin D2 levels. Stoichometric assays suggest that TFIID contains two molecules of TAF4, and it is possible that one TFIID complex could contain both TAF4 and TAF4b. TAF4 and TAF4b may be working together to regulate granulosa cell gene expression. ChIP assays have determined that both TAF4 and TAF4b are present at the promoters of cyclin D2

and c-Jun [5]. If TAF4 is compensating for loss of TAF4b in the ovary, it is doing so without increasing its own levels above wildtype. The relative contributions of TAF4 and TAF4b to ovary development and function are not known. What is clear is that TAF4b has at least one very important function in fertility that TAF4 is not able to replace.

Evolution of TAF4b

While TAF4 is widely conserved across all metazoans, TAF4b is most highly conserved among members of Amniota (mammals, birds and reptiles). TAF4b also has a homolog in the frog but as of yet, none have been identified in monotremes, marsupials or fish. Interestingly, the testis specific *Drosophila* tTAF4s have taken a separate evolutionary path. *No hitter* is more similar to Drosophlia TAF4 than mouse TAF4b. Thus, evolution of gonadal functioning TAF variants have arisen multiple times, each leading to genes with specific roles in fertility. So far gonadal variants of TAF4, TAF5 and TAF7 have been identified in mouse, and additionally, a human-specific TAF1 testis variant has been discovered. TAFs appear particularly amenable to gene duplication events resulting in new activity, contributing to sexual reproduction.

A recent study on the evolution of tTAFs has revealed that the 5 testis-specific TAFs all arose within a relatively short time of each other and that each TAF-duplication was an independent event [16]. The mechanism of the duplication events is not clear, although the typical mode of retrotransposition seems unlikely due to the presence of introns in TAF variants. However, transposition coming from an incompletely processed transcript cannot be ruled out and given the high rate of recruitment of retrotransposed genes in the male germline, this scenario is likely. Unlike TBP and the general TAFs,

which evolve slowly, the tTAFs evolve rapidly- a phenomenon that is especially prevalent with testis genes. There is a bias towards testis expression of the fastest evolving genes in *Drosophila* , which may lead to enhanced gonadal function and/or speciation [16]. Although these duplication events are not as common in the ovary as in the testis, a number of newly evolved genes also show enriched expression in the ovary [16]. It is possible that in the case of TAF4b, the gene may have arisen as a consequence of the rapid evolution of testis-expressed transcripts, but gave rise to either a novel, shared function in males and female gonads, or developed individual roles in each sex.

Ovarian-specific requirement for TAF4b in female mice

It may be that much of what we have learned about TAF4b is not at the base of how it is functioning in fertility. Perhaps what we have observed so far regarding its role in gene expression is residual activity either from before it diverged from TAF4, or activity that has accompanied its neo-functionalization, but is not what is underlying infertility in its absence. We have observed increases in TAF4b levels in the liver, B-cells and granulosa cells during periods of proliferation, yet in TAF4b-null mice, these proliferation events are not impaired. Although granulosa cell proliferation is severely reduced upon hormonal stimulation, we still see large ovarian follicles that have many layers of granulosa cells in TAF4b-null, unstimulated mice up through 8 weeks of age.

By using hematoxylin and eosin staining of ovarian sections to more precisely document the degeneration in the TAF4b-null ovary that takes place within the first few months of life, we are now able to make more informed choices when designing experiments. Because there is such a rapid loss of follicles, the age at which we obtain ovary samples can profoundly affect the outcome of our experiments. Although we knew

that the TAF4b-null female phenotype worsened with age, we did not know the extent to which this was true. To our surprise, we could not visually distinguish between TAF4b-null and wildtype ovary sections until about postnatal day (pnd) 63. This was curious to us because TAF4b-null mice have elevated FSH levels by pnd 23 and go in to persistent estrus by approximately pnd 50. Our previous characterization of follicles between TAF4b-null and wildtype ovaries revealed that even though primordial follicles are reduced in the TAF4b-null ovary the recruitment from primordial to primary follicle stages is not significantly lowered [8]. Taken together, it appeared that the TAF4b-null mouse was experiencing premature ovarian senescence possibly due to a rapid depletion of oocytes.

Although reproductive capacity diminishes with mammalian female age, this can not fully explain the absolute infertility associated with loss of TAF4b. Previous reports have shown that TAF4b-null oocytes have meiotic defects as well as zygotic cleavage problems that result in arrest at or before the 2-cell stage [11]. In addition, there is an excess culling of primordial follicles in TAF4b-null ovaries between pnd 0 and 3. It is possible that many of the fertility defects that we observe are due to a problem within the TAF4b-null oocyte.

If the initial quality of the TAF4b-null oocyte was decreased, it may explain why approximately 50% more primordial follicles are lost during the initial period of cyst break down. If, in fact, this culling process represents a quality control mechanism that chooses the healthiest follicles to remain, perhaps there are fewer TAF4b-null oocytes that are up to standard. In support of this notion, a recent study using human oocytes has proposed that TAF4b is a marker of oocyte quality [6]. Although there is plenty of

evidence to attribute TAF4b-null infertility to an oocyte defect, without molecular evidence, there is no way of knowing if the oocyte is defective due to transcriptional misregulation within the germ cell, if mis-expression in other cell types is leading to the oocyte problems, or if it is a combinatorial effect.

Oocyte development is heavily reliant on the combined support of other ovarian cell types and the hypothalamic-pituitary-ovarian axis. Transcriptional disruption in the granulosa cells or in the pituitary could certainly manifest itself as a defective oocyte. To address these possibilities we examined gene expression in the pre-pubertal mouse, before obvious ovary deterioration has taken place. At 3 weeks of age we observed normal expression of gonadotropin subunits, inhibin βB and the pituitary Foxo protein, Foxl2, but reduced expression of follistatin in the pituitary TAF4b-null mice. These data suggest that the elevated FSH level could be due to mis-regulation of follistatin, which would lead to activation of the pituitary by activin. Follistatin is also reduced in the ovaries of TAF4b-null mice, indicating that TAF4b is required for the proper expression of follistatin in both the ovary and pituitary at 3 weeks of age. Although our histological sections of TAF4b-null mice appear equivalent to those of wildtype at 3 weeks, the data obtained from our measure of estrous cyclicity prompted us to re-evaluate follistatin levels. On average, our wildtype mice experience the onset of estrous at about 30 pnd, TAF4b heterozygotes at 29 pnd and TAF4b-null mice at 24.5 pnd. Before the onset of puberty, it is important for gonadotropin levels to remain low, but once puberty has initiated, the levels of circulating hormones need to be dynamic [17]. Because the onset of puberty is earlier for TAF4b-null mice, the reduced follistatin we see at 3 weeks may simply be an indication that the protective pre-pubertal level of follistatin has been lifted.

Examining levels of follistatin at 2 weeks of age revealed that follistatin levels are equivalent between TAF4b-null and heterozygous mice and that reduced follistatin is not likely to be the cause of elevated FSH in the TAF4b-null mouse, but an effect of early entry to estrous.

To obtain a more comprehensive understanding of gene expression in the TAF4b-null ovary that may be underlying the degeneration beginning at 8 weeks, we performed a microarray using wildtype, heterozygous and TAF4b-null ovaries from pre-pubescent 3-week old mice. A previous microarray had been done during the initial characterization of TAF4b-null females using hormonally stimulated adult ovaries [14]. Since then, we have learned that TAF4b-null mice do not respond well to hormonal stimulation, and that there is a near absence of granulosa cells in the adult ovary whereas the cell-type proportions at 3 week appear nearly equivalent. The data from the initial microarray revealed downregulation in numerous granulosa cell genes. Taking into account what we know now, the reduction in those transcripts is likely a reflection of reduced granulosa cells in the TAF4b-null ovary, compounded by the reduced ability of those granulosa cells to divide upon hormonal stimulation. Our aim with the microarray at 3-weeks of age was to establish new target genes that are mis-regulated and may be contributing to the defects observed in the adult ovary.

The changes in gene expression between TAF4b genotypes overall, were subtle. Of the genes with significant changes between TAF4b-null and wildtype ovaries ($p < 0.05$), 25 genes were expressed in the TAF4b-null ovary at levels 1.5-fold greater than wildtype, while 60 genes were reduced 1.5-fold or more. Functional analysis revealed that the downregulated genes fell into 2 main groups- the Obox family of oocyte specific

transcription factors and genes that code for proteins with nucleotide binding properties. An interesting group of genes that was reduced in this microarray is the NALP family. We saw reduction of several NALP genes with the most prominent reductions observed in NALP4e, NALP9c, NALP9a and NALP4b. While this family of genes has not been extensively characterized there is much support for their roles in female fertility.

The NALP family, identified in the ovary during a screen for genes involved in oocyte aging, is composed of 14 members in humans and 20 in mice [18, 19]. Of the 14 NALP members, 12 were detected in human oocytes and pre-implantation embryos, and seven were detected in sperm. Eight human NALPs (NALP 4, 5, 8, 9, 11, 12, 13, and 14) showed a similar expression pattern with high expression levels in oocytes then decreasing progressively in embryos, resulting in very low levels in day 5 embryos, suggesting a developmental role for NALPs prior to implantation [20]. NALP5, which was not significantly reduced in our microarray, is also known as MATER, and female mice null for this gene are infertile with embryos arresting at the 2-cell stage [21].

Some NALP members also participate in innate immunity with their incorporation into a large multi-subunit complex called the inflammasome [22]. Although reports have demonstrated roles for only NALP1, 2 and 3, the domain structure between NALP proteins is very similar. The pathogen sensing mechanism for NALPs is not well understood and only the activating ligands for NALP3 have been determined- NALP3 recognizes bacterial RNA, ATP and uric-acid crystals [23]. Upon pathogen detection, NALPs recruits ASC (apoptosis-associated speck-like protein containing a CARD) via homotypic PYRIN domain interactions, which in turn, recruits caspase 1 (or 5), leading to the activation of interleukin (IL)-1β and IL-18 processing [19]. Mutations in NALP3

have been linked to abnormal inflammasome activation causing periodic fever and aberrant inflammasome activation has also been linked to autoinflammatory diseases including gout [19]. If there is a relationship between the inflammasome activity of NALPs and their roles in reproduction it has not been reported.

There has, however, been research done on the role of the IL-1 system in ovarian function. The act of ovulation is an inflammatory process and IL-1 is involved in the signaling events leading up to ovulation. The ovary produces IL-1 system component proteins (ligands IL-1α, IL-1β, IL-1 receptor antagonist, and IL-1 receptors) in multiple ovarian cell types [24]. IL-1β mRNA has been detected in rodent mural and cumulus granulosa cells, theca cells and in the oocyte. There have been numerous reports of IL-1β activity within the follicle that implicate it in oocyte maturation, cumulus cell expansion, and the inflammatory events of ovulation [24, 25]. There have also been correlations drawn between intrafollicular levels of IL-1 and the quality of the oocyte, as indicated by the health of embryos resulting from *in vitro* fertilization [26, 27]. In humans IL-1β mRNA and proteins have been detected in embryos at the time of fertilization, suggesting their presence in the mature oocyte [28].

In our microarray the levels of IL-1β mRNA were similar between TAF4b-null and wildtype ovaries. At 3 weeks of age, however, we do not yet observe pre-ovulatory follicles. If the reduction of the NALP genes leads to reduction of IL-1β, we would not expect to see a difference until the time IL-1β mRNA is normally induced.

In addition to the NALPs, we also identified another gene that is reduced in 3 week-old TAF4b-null ovaries, Mov10l1. Of all the genes in the array, Mov10l1 showed the most reduction from wildtype – levels were reduced 6-fold as determined by qRT-

PCR in TAF4b-null ovaries compared to wildtype at both 3 and 2 weeks of age, indicating that unlike follistatin, its reduction was probably was not an effect of early onset of estrous. A follow-up assay examining Mov10l1 expression in GC1 cells showed a specific reduction upon TAF4b siRNA-mediated knockdown, while knockdown of TAF4 resulted in increased Mov10l1 expression. TAF4b-dependence is unique to Mov10l1 and its paralog, Mov10, is neither reduced in TAF4b-null ovaries, nor reduced upon knockdown of TAF4b.

Possible role for Mov10l1 in germline-specific RNAi

Mov10l1 has not been well characterized in mammals. Most of the studies on this putative RNA helicase have been on its two splice variants, Csm and CHAMP, which are involved in heart development [29, 30]. The full length Mov10l1 was previously identified in as a testis-germ-cell-specific gene [31], but since then ESTs have been identified in oocytes of mouse and frogs [32, 33]. The most interesting aspect of finding reduced levels of Mov10l1 in TAF4b-null ovaries is that this gene shares a very high degree of homology with the *Drosophila* protein, Armitage (Armi) [34].

Disruptions in *armitage* lead to fertility defects in both males and females due to RISC maturation failure [35]. *Armitage* was first identified in a screen to find maternal effect proteins that are required for proper axis specification in *Drosophila* . Because of the homology of *armitage* to the Arabidopsis protein SDE3, which is involved in post-transcriptional gene silencing in plants, Zamore *et al.* investigated its role in the related process in *Drosophila* , RNAi. Back to back publications in a 2004 issue of Cell by Cook *et al.* and Tomari *et al.* characterized *armitage*-mutant defects in both males and females and document the bulk of our knowledge about Armi [35, 36]. *Armitage* was found to be

necessary for silencing of *stellate* in the *Drosophila* testis through RNAi and oocyte lysates from *armitage*-mutant ovaries are defective in RNAi. Specifically, *armitage* is required for the later steps of assembling siRNA into a functional RISC.

With the highest expression of *armitage* in the germline, and no developmental defects in *armitage*-mutant flies aside from infertility, the defects we observe in TAF4b-null mice might arise from reduced level of Mov10l1. Armi is first expressed in *Drosophila* in early oogenesis and is responsible for silencing of *oskar* until repression is lifted when it is properly localized in mid-oogenesis. In *armitage*-mutant flies, the transcript levels of *oskar* were equivalent to the wildtype, but it was aberrantly translated causing premature accumulation of Osk protein. Oocyte axis specification was also disrupted in *armitage* mutants. Patterning defects do not arise from mis-expression of Osk, but rather, disruption of the RNAi system causes polarization defects in early oogenesis leading to microtubule disorganization.

The main assay we have used to detect differences between wildtype and TAF4b-null ovaries has been qRT-PCR. If there were defects in gene silencing, this might not be very apparent when measuring mRNA levels. *Drosophila* oocytes experience problems during early oogenesis from loss of Armi and we have never checked embryonic oocyte development in the mouse, which may be when critical Mov10l1-dependent events occur to set up the ovary for postnatal function.

Of the TAF4b-null oocytes that develop enough to be fertilized, they cannot progress past the 2-cell stage and the cleavages are not symmetrical [11], which could result if axis specification was disturbed as in *armitage* mutants. The *armitage*-mutant ovaries are reported to display multiple defects stemming from mis-regulated RNAi. The

extent to which this particular method of RNAi processing is conserved from *Drosophila* to mammals is not known.

When assessing the fertility defects in gene mutants with both male and female infertility, it is often beneficial to compare phenotypes to determine how the disrupted gene regulates normal fertility. In the case of *armitage* mutation in *Drosophila* , the reproductive defect in males is thought to arise from Stellate (Ste) protein accumulation. Two hypomorphic mutant *armitage* alleles of different strengths were assayed for *stellate* silencing. One allele was found to have much more Ste protein in the testis, indicating severe impairment in RNAi-mediated silencing, while the other allele resulted in an intermediate amount of Ste. In breeding assays using wildtype females, the progeny from wildtype males yielded 97% hatched embryos in comparison to crosses with the intermediate *armitage* mutant, which yielded 75% hatched, and the more severe *armitage* mutant, which yielded about 45%. *In vitro* RNAi assays performed in the ovary lysates from the two *armitage* mutants confirmed that RNAi was more severely impaired in one of the mutants, with the other having an intermediate impairment. The same *armitage* alleles in females resulted in hatch rates of 0% for both the severe and intermediate mutations [35, 36]. These sex-specific levels of infertility, although not exactly the same as those found in TAF4b-null mice, are strikingly similar given the fundamental differences in reproduction between flies and mice.

Mov10l1 is reduced in both male and female TAF4b-null gonads during embryogenesis (data not shown) and postnatally compared to wildtype. Endogenous miRNAs are emerging as critical factors in numerous biological processes including normal gonad development and fertility, but also in gonadal pathologies [37, 38].

Additionally miRNAs have been shown to be involved in germline stem cell renewal [39]- the underlying disruption of which is believed to lead to TAF4b-null male infertility. Although the reproductive defects observed in TAF4b-null mice are likely caused by multiple transcriptional disturbances, it is possible that the underlying cause of male sub-fertility and female infertility results from mis-regulation of a specific germline miRNA pathway due to a reduction in Mov10l1.

The next step in understanding how TAF4b plays its essential role in fertility is to characterize Mov10l1 in mammalian gonads. We have shown that Mov10l1 expression is two orders of magnitude higher in the testis than in the ovary so a logical starting place for the study of this protein is the testis. To determine the extent to which Mov10l1 is expressed in a germ cell-specific manner in mice, we would perform *in situ* hybridization or immunofluroescent detection in wildtype testis sections. No Mov10l1 antibodies are commercially available at this time, however one exists that recognizes Armi. The polyclonal Armi antibody was raised against the C-terminus of the *Drosophila* protein, which shares about 60% homology with Mov10l1, but that region is also similar to Mov10, with which Armi shares 53% homology. Since Mov10l1 appears to be expressed at levels far above Mov10 in the testis, perhaps cross reaction would not pose a great problem. Optimally we would produce our own Mov10l1-specific antibody which could be used in a number of assays to determine how Mov10l1 functions *in vivo*.

Elegant experiments have been performed in *Drosophila* that determined that armitage acts specifically in the ATP-dependent incorporation of single stranded siRNA into RISC [35, 36]. Because armitage does not bind the RISC complex, co-immunprecipitation of Mov10l1 would not be expected to be pulled down with other

RISC components and validate its involvement in mammalian RNAi. RISC assembly, however, should be a read-out of Mov10l1 function if armitage and Mov10l1 are acting in the same manner. If we can establish a mouse ovary lysate similar to the *Drosophila* ovary lysate in which to test RNAi, we can compare both RISC assembly and target gene cleavage between TAF4b-null and wildtype ovary lysates.

Post-transcriptional gene regulation is a mechanism used in both somatic and germ cells. In somatic cells, peri-nuclear structures called processing bodies, or P-bodies, harbor the protein and RNA components involved in translational repression and mRNA degradation [40]. An analogous, but specialized, structure is found in testicular germ cells called the chromatoid body, which contains general processing factors, such as the Argonaute family proteins, as well as germ-cell specific factors [41, 42]. Chromatoid and P-bodies are sites where tight but potentially transient control of protein expression occurs by repressing translation in a miRNA-specific manner; the repressed transcripts can be released and translated when there is a physiological requirement [43, 44].

During spermatogenesis there is a high degree of transcriptional activity necessary both for mitotic growth of spermatogonia and for production of mRNA that will be repressed until after the chromatin compaction events late in spermatogenesis which prevent transcription [43]. Work in *Drosophila* has implicated translational repression in oocyte development and, specifically, miRNA-mediated translational control, in the production of a healthy oocyte [36, 45]. As of yet there has not been a mammalian female equivalent of the chromatoid body identified, but given that miRNAs and translational control play an important role in oogenesis, this process is well worth investigating.

Interestingly, Mov10, which shares a high degree of homology with Mov10l1 was found to be localized to P-bodies and required for miRNA guided mRNA cleavage in HeLa cells [46]. Of the mouse tissues we tested for Mov10 and Mov10l1 expression, Mov10 was highest in the spleen and not enriched in the testis. Mov10l1, however, is very highly enriched in the testis, lending further support for its role in germ cell-specific translational control. Because of the inherent differences in germ cell development between males and females, it is possible that Mov10l1 is just as important in female germ cell development, yet we do not observe the same degree of enrichment in the postnatal samples we collect. This could be due to the differences in timing of germ cell development and perhaps Mov10l1 is acting during fetal or neonatal oogenesis as opposed to postnatal spermatogenesis. Additionally, there is an exponentially higher concentration of germ cells in the testis compared to the ovary. If Mov10l1 is specifically enriched in germ cells, the 100-fold reduction we observe from the levels in testis to ovary may not be indicative of a reduced requirement. It is possible that if Mov10l1 reduction in TAF4b-null gonads results in infertility it may be through distinct mechanisms in the testis and the ovary although it is likely that post-translational gene silencing will be disturbed in both sexes. Here, we have proposed a link between transcriptional control of germline development by TAF4b with post-transcriptional gene silencing in the mammalian ovary.

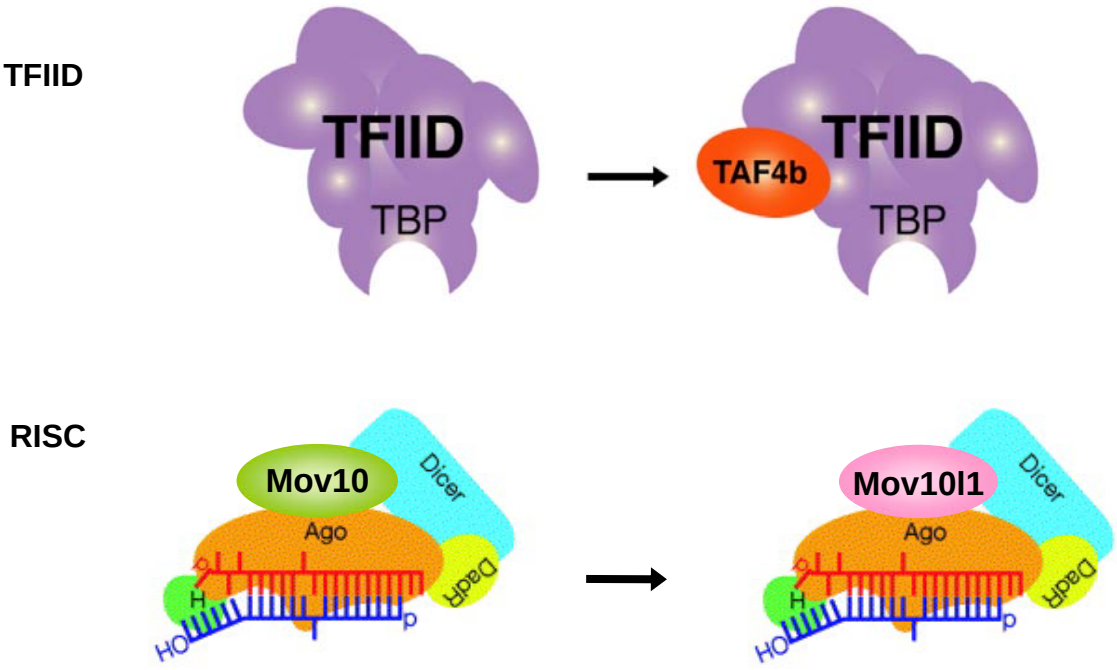

Figure 1. Model of germ cell variation of global regulatory complexes. The mammalian TFIID complex incorporates a gonadal-enriched TAF variant, TAF4b, which is required for fertility. Similarly the RISC complex may use Mov10l1 to carry out germ-cell specific processes that Mov10 is unable to perfom.

Summary

The studies presented here highlight the specific requirement for TAF4b in female mammalian fertility. Although TAF4b is differentially regulated in other tissues, there is no evidence to support its requirement outside of the gonad. We have shown that TAF4b directs both granulosa cell and oocyte-specific gene expression. Deletion of TAF4b leads to multiple ovarian deficiencies and accelerated depletion of follicle reserves, mimicking the human condition of Primary Ovarian Insufficiency. We have introduced a new set of putative TAF4b-target genes that are functionally significant to human reproduction and may underly yet unidentified germline-specific processes. We have also identified a TAF4b-regulated germ cell-specific RNA helicase, Mov10l1, which may be involved in a novel mammalian RNAi pathway.

Although preliminary studies have implicated TAF4b in extra-gondal proliferation, we have shown that it is not a general requirement for cell cycle progression in differentiated tissues. TAF4b does not appear necessary, despite consistently elevated expression, in proliferating cells. Similarly, TAF4b has been localized to a number of specific gene promoters, but we show here that TAF4b is not an absolute requirement for their normal expression *in vivo*. What we have observed during the characterization of this variant TAF may be residual activity carried through evolution, but not relevant to its essential function in the mammalian gonad.

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