Nucleoporin107 mediates female sexual differentiation via Dsx

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Abstract

We recently identified a missense mutation in Nucleoporin107 (Nup107; D447N) underlying XX-ovarian-dysgenesis, a rare disorder characterized by underdeveloped and dysfunctional ovaries. Modelling of the human mutation in *Drosophila* or specific knockdown of Nup107 in the gonadal soma resulted in ovarian-dysgenesis-like phenotypes. Transcriptomic analysis identified the somatic sex-determination gene doublesex (*dsx*) as a target of Nup107. Establishing Dsx as a primary relevant target of Nup107, either loss or gain of Dsx in the gonadal soma is sufficient to mimic or rescue the phenotypes induced by Nup107 loss. Importantly, the aberrant phenotypes induced by compromising either Nup107 or *dsx* are reminiscent of BMP signaling hyperactivation. Remarkably, in this context, the metalloprotease AdamTS-A, a transcriptional target of both Dsx and Nup107, is necessary for the calibration of BMP signaling. As modulation of BMP signaling is a conserved critical determinant of soma-germline interaction, the sex and tissue specific deployment of Dsx-F by Nup107 seems crucial for the maintenance of the homeostatic balance between the germ cells and somatic gonadal cells.

Introduction

Germline-soma communication lies at the heart of proper gonad development, and thus is essential for formation and coalescence of the primitive embryonic gonad until generation of the adult gonad (1-3). Gonadogenesis also relies upon coordination between autonomous and non-autonomous cell mechanisms that direct correct specification, patterning, and subsequent morphogenesis (4, 5). Furthermore, the developmental program culminating in proper gonad formation must integrate both non-sex-specific ‘housekeeping’ functions and sex-specific signals to establish and maintain sexually dimorphic traits. Therefore, functional aberrations that affect individual molecular components, either sex-specific or non-sex-specific, result in various clinical disorders and infertility.

XX ovarian dysgenesis (XX-OD) is a rare, genetically heterogeneous disorder that is characterized by underdeveloped, dysfunctional ovaries, with subsequent lack of spontaneous pubertal development, primary amenorrhea, uterine hypoplasia, and hypergonadotropic hypogonadism (6). We recently identified a recessive missense mutation in the nucleoporin-107 (*Nup107*) gene (c.1339G>A, p.D447N) as the causative mutation for isolated XX-OD (without other developmental deficits) in five female cousins from a consanguineous family (7). All men in the family had normal pubertal development and those married have multiple children.

Nup107 is an essential component of the nuclear pore complex, enabling both active and passive transport in every nucleated cell. In light of this ubiquitous and vital function, the sex-specific and tissue-restricted nature of the *Nup107* XX-OD phenotype is highly intriguing. Taking into account the high conservation of the aspartic acid residue altered by the mutation, we used a *Drosophila* model to assess *Nup107* function in human female gonadal development. Our previous studies demonstrated that RNAi-mediated knockdown (KD) of *Nup107* in somatic gonadal cells led to female-specific sterility due to defective oogenesis, while male flies developed normally and remained fertile (7). Furthermore, generating a *Drosophila* model of the human mutation by introducing an *RFP-Nup107^{WT}* transgene in *Nup107*-null flies rescued both lethality and fertility of female flies; however, introduction of an *RFP-Nup107^{D364N}* transgene recapitulating the familial XX-OD mutation, while rescuing lethality, resulted in severely reduced female fertility, ovariole disintegration, and extensive apoptosis (7).

A second mutation in *Nup107* (c.1063C>T, p.R355C) has since been independently identified as a cause of XX-OD (8). Notably, our analysis had predicted salt bridge interactions between the altered Aspartic Acid 447 in our study and the Arginine 355 altered in the second family, strengthening the notion that *Nup107* performs an essential and conserved ovary-specific function during female gonadogenesis. Here, to uncover the molecular underpinnings of *Nup107*'s function, we analyzed the 'loss of function' phenotypes of *Nup107* at both the cellular and transcriptional levels.

Results

***Nup107* mutant ovaries display an ovarian dysgenesis like phenotype**

A closer inspection of *Nup107*^{D364N} adult ovaries revealed that the female gonads displayed phenotypic traits closely resembling human ovarian dysgenesis. Specifically, the phenotypes ranged from rudimentary, small ovaries with fewer ovarioles to bilateral dysgenesis where both ovaries were completely absent (Figure 1B-D). Approximately 33% of *Nup107*^{D364N} ovaries were either under- or non-developed, in comparison to milder defects in only 6% and 5% in *Nup107*^{WT} and *yw* control flies, respectively (Figure. 1A, G). We found similar ovarian defects when inserting the *Nup107* mutation (1090G >A, p.D447N) into the *Drosophila* genome using CRISPR (9).

To discern if *Nup107* is necessary in the somatic or germline component of the gonad, or in both, we selectively inactivated *Nup107* function using *UAS-Nup107-RNAi* in combination with either a somatic gonadal specific driver *traffic jam-Gal4* (*tj-Gal4*) or a germ cell specific driver (*nanos* i.e., *nos-Gal4*). Interestingly, only soma specific knockdown of *Nup107* recapitulated the mutant phenotype, where 51% of adult ovaries were 'dysgenic' or under-developed (Figure. 1E-G). Supporting the conclusion that in this functional context *Nup107* is required primarily in the soma, germline specific inactivation of *Nup107* did not lead to significant phenotypic abnormalities compared to the control (9%, Figure 1G).

Figure 1. Phenotypic characterization of the ovaries compromised for *Nup107*. (A) *yw* control *Drosophila* ovaries. In contrast, *Nup107^{D364N}* ovarian samples display a variety of aberrations, including (B) small or (C) shriveled ovaries, and (D) bilateral dysgenesis. Knockdown of *Nup107* using *tj-Gal4* driver recapitulated the same phenotypes as *Nup107^{D364N}*, with flies exhibiting (E) underdeveloped or (F) non-developed ovaries. (G) The percentages of under/non-developed ovaries in *yw* (n=196), *Nup107^{WT}* (n=504), *Nup107^{D364N}* (n=890), *tj>Nup107* (n=316), and *nos>Nup107* (n=64) flies.

***Nup107* mutant ovaries display increased BMP signaling activity away from the germline stem cells (GSC) niche**

To understand the functional underpinnings of the ovarian phenotypes induced by the D364N mutation we sought to analyze the *Nup107* mutant ovaries carefully. The *Drosophila* ovary is made up of 16-20 ovarioles that function individually as egg production lines. The germarium, situated at the anterior tip of the ovariole, contains the somatic stem cell niche and adjacent Germline Stem Cells (GSCs) (10, 11). Typically, a GSC divides asymmetrically to generate another stem cell and a cystoblast. Cystoblasts divide and differentiate to form sixteen interconnected cystocytes, with the dynamically expanding fusome acting as the connecting link (Figure. 2A) (12, 13). Thus, GSCs can be readily identified by the presence of a round fusome, whereas their differentiated daughter cells display extended, branched

123 fusomes. Ovary staining revealed that unlike *yw* and *Nup107^{WT}* germaria, which
124 contained 2-3 spherical fusomes, *Nup107^{D364N}* germaria contained an average of 6
125 spherical fusomes per germarium (7% of *Nup107^{WT}* vs. 34% of *Nup107^{D364N}*; Figure.
126 2B- D). Similar results were seen in germaria where *Nup107* expression was knocked
127 down (KD) using the somatic gonadal driver *tj-Gal4* (hereafter *tj>Nup107 KD*;
128 Figure. 2B, E). Thus, *Nup107^{D364N}* germaria contain elevated numbers of cells with
129 spherical fusomes, characteristic of both GSCs and undifferentiated cystoblasts.
130 Interestingly, an increase in the total number of undifferentiated germ cells is
131 reminiscent of hyperactivation of the Bone Morphogenetic Protein (BMP) signaling
132 pathway (14).

133 We thus wondered if inappropriately expanded BMP signaling is responsible
134 for the failure in GSC differentiation. The somatic terminal filament (TF) and cap
135 cells, which together constitute the GSC niche, normally secrete a BMP ligand,
136 Decapentaplegic (Dpp) (15, 16). Binding of Dpp to its cognate receptor Thickveins
137 (Tkv) expressed in GSCs triggers a signal transduction cascade, which ultimately
138 represses the expression of the master differentiation gene *bag of marbles* (*bam*) in
139 the GSC region (17). As GSC daughter cells move away from the niche, BMP
140 signaling is weakened, resulting in the induction of Bam expression. We found that
141 Bam expression in mutants was continuously repressed and completely absent in 43%
142 of *Nup107^{D364N}* and 42% of *tj>Nup107 KD* stage 2a germaria, compared to 6% and
143 11% in *yw* and *Nup107^{WT}* flies, respectively (Figure. 2B, F-I). As phenotypic
144 consequences resulting from *Nup107* loss seemed analogous to those induced by
145 excess BMP signaling, we sought to analyze the downstream components of the BMP
146 pathway.

147 The transcriptional response to the BMP signal that emanates from the stem
148 cell niche is mediated by phosphorylation of MAD (pMad) which translocates to the
149 nucleus and with its binding partner, Medea, regulates pathway targets (17-19). pMad
150 represses *bam* and thus allows for maintenance of the undifferentiated state and self-
151 renewal of GSCs (20). We found that pMad levels in *Nup107*-compromised germaria
152 are abnormally elevated in regions distant from the GSC niche. Specifically, 50% of
153 *Nup107^{D364N}* and 57% of *Nup107 KD* germaria show high expression of pMad,
154 compared to 7% of *yw* and 17% of WT-rescued germaria (Figure. 2B, F-I). Taken
155 together these findings support the notion that compromising *Nup107* activity leads to
156 hyperactivation of BMP/Dpp signaling away from the GSC niche and suggest that

Nup107 restricts the range and/or strength of Dpp signaling required for proper differentiation of the GSCs.

Figure 2. *Nup107^{D364N}* adult ovaries demonstrate increased germ line stem cell number (A) A scheme of the *Drosophila* gerarium. (B) Quantification of spherical fusomes (n=57, 74, 100, 27), Bam (n=127, 137, 139, 64) and pMad (n=122, 121, 225, 48) phenotypes in *yw*, *Nup107^{WT}*, *Nup107^{D364N}* and *Nup107* KD, respectively. (C) *Nup107^{WT}* germaria contain 2-3 cells with spherical fusomes, indicated by arrowhead, while (D) *Nup107^{D364N}* and (E) *tj>Nup107* germaria contain an average of 6 cells with spherical fusomes. (F) *yw* and (G) *Nup107^{WT}* germaria show normal pMad (blue) and Bam (green) expression compared to (H) *Nup107^{D364N}* and (I) *tj>Nup107* germaria which show reduced Bam levels and expanded pMad expression.

Impairment of the GSC progeny differentiation niche in *Nup107* mutants

Escort cells (ECs) and their cellular processes, which encapsulate the GSC daughter cells that leave the stem cell niche, constitute a niche that controls germ cell differentiation. This is thought to be achieved, in part, by restricting BMP signaling (21, 22). ECs utilize Hh, Wnt, EGFR, and Jak-Stat signaling to prevent BMP signaling in GSC progeny (23-32). Many of these signals act to sustain the

cytoskeletal structure of the processes that emerge from the ECs. These processes were shown to be essential for the proper differentiation of GSC progeny. The accumulation of undifferentiated GSCs and the expanded range of BMP signaling observed in *Nup107* compromised germaria could result from the failure of the EC differentiation niche function. We reasoned that such a failure may be reflected in the cellular extensions emerging from the ECs. To test this idea, ovaries were co-stained with anti-Traff Jam (TJ) and anti-Coracle (Cora). Cora is a structural protein that is highly expressed in the cellular extensions of ECs (32, 33) whereas TJ is a transcription factor expressed in the nuclei of ECs and follicle stem cells in the germarium (34). The cellular extensions of the ECs in *yw* and *Nup107^{WT}* germaria were readily detected (Figure. 3A, B). In contrast, these cellular extensions were either dramatically reduced or completely lost in over 36% and 48% of the *Nup107^{D364N}* and *tj>Nup107 KD* germaria, respectively (Figure. 3C-E). It should be noted that the similar number of escort cells were present in both WT and *Nup107* compromised germaria. Together these data imply that compromising *Nup107* impairs the formation of cellular extensions in ECs required for restricting the BMP signal in the GSC differentiation niche.

Figure 3. Adult ovaries demonstrate few or no ECs extensions. In germaria taken from (A) *yw* and (B) *Nup107^{WT}*, the ECs extensions were easy to note. In contrast, ECs extensions from (C) *Nup107^{D364N}*, (D) *tj>Nup107 KD*, (E) *tj>dsx KD* and (F) *tj>adamTS-A KD* germaria were dramatically reduced or lost. (G) Quantitation of Coracle extensions from *yw* (n=88), *Nup107^{WT}* (n=32), *Nup107^{D364N}* (n=25), *tj>Nup107 KD* (n=65), *tj>dsx KD* (n=60) and

tj>adamTS-A KD (n=54). Anti-Cora and anti-TJ staining are shown in green and magenta respectively.

Soma-germline homeostasis in the larval gonad requires Nup107

To trace back the phenotypic consequences of Nup107 loss to the earlier stages of gonad development, we sought to analyze the third instar larval stage (LL3) gonads. At this stage, the *Drosophila* larval gonad consists of a somatically derived stem cell niche and its adjacent primordial germ cells (PGCs, Figure. 4A). Interspersed amongst the PGCs are their somatic support cells, known as Intermingled Cells (ICs). We paid special attention to the ICs as they serve as the progenitors of the ECs. Dissection of *yw*, *Nup107^{WT}* and *Nup107^{D364N}* larvae showed that their gonads were equally present and readily identifiable in all three genotypes (Figure 3-Figure Supplement 1).

To assess if the somatic and germline components of the larval gonad are specified and patterned correctly in a Nup107 compromised background, we stained larval gonads from both *Nup107^{D364N}* flies and those with somatic KD of Nup107 (*tj>Nup107 KD*), using antibodies which specifically mark the PGCs (VASA) and ICs (TJ). Confocal imaging of these larval gonads revealed that both cell types are present at this developmental stage, however their spatial organization appeared to be disrupted. Moreover, both *Nup107^{D364N}* and *tj>Nup107 KD* larval gonads contained excess numbers of PGCs (Figure. 4D, E) which formed large clusters devoid of ICs (Figure. 4E', I, J). While the total number of mutant ICs was unaffected (Figure 4-Figure Supplement 1), these cells failed to intermingle with the PGCs and often remained clustered at the periphery of the gonadal tissue (Figure. 4E-H). Staining with antibodies against an adducin-like molecule (1B1) which marks the fusome, a sub-cellular organelle, revealed elevated numbers of cells with spherical fusomes, normally characteristic of germline stem cells (Figure 3-Figure Supplement 1 B, D). Proper soma-germline communication is necessary for the maintenance of the homeostatic balance required for adequate proliferation and differentiation of both cell types. Altogether these data show that loss of *Nup107* in the somatic gonadal precursors affects the soma-germline communication, adversely affecting the total PGC count and relative positioning of the ICs.

Figure 4. *Nup107*^{D364N} larval gonads display aberrant cellular number and arrangement. (A)

Schematic representation of the LL3 larval gonad with its different cell types. (B) Confocal section of *Nup107*^{WT} gonad containing on average 18 PGCs (VASA, green), compared to (C) 27 in *Nup107*^{D364N} gonads. (D) Quantitation of the total PGCs per confocal section in each gonad, in yw, *Nup107*^{WT}, *Nup107*^{D364N}, and *Nup107* KD (n=42, 71, 88, 22). (E) *Nup107* KD larval gonads contain excess PGCs (VASA, green) and abnormally dispersed ICs (TJ, blue). *Nup107*^{D364N} larval gonads exhibit a range of IC dispersion patterns from (F) normal to (G) moderate to (H) severe. (I) The percentage of each IC phenotype found in yw, *Nup107*^{WT}, *Nup107*^{D364N} and *Nup107* KD gonads (n=22, 61, 58, 21). (J) Representation of the number of PGCs per gonad missing an immediately adjacent IC, due to abnormal dispersion.

Sex-determination gene *Dsx* is a target of *Nup107*

We thus sought to explore if the *Nup107* D364N mutation influences transcription in the female larval gonads and if so, whether the possible changes in the transcriptional profile could be correlated with the developmental defects observed in *Nup107* mutant ovaries. To assess the genome-wide transcriptional changes in the *Nup107* mutant ovaries, we performed an unbiased transcriptome analysis on yw,

Nup107^{WT} and *Nup107*^{D364N} LL3 larval gonads using RNA-seq (GEO accession number GSE141094). We identified 82 candidate genes (Tables supplement 1, 2) which displayed significant changes in mRNA expression in the larval gonad upon compromising *Nup107*. Among these candidates, we were particularly intrigued by the decreased expression of the DMRT transcription factor family member *doublesex* (*Dsx*), which is critical for sex-specific differentiation (35-37). We confirmed that *dsx* is a target of *Nup107* using qRT-PCR (Figure 4-Figure Supplement 2).

Dsx is expressed in both sexes, but is regulated via sex-specific alternative splicing, which results in the generation of either female (*Dsx*^F) or male (*Dsx*^M) specific isoforms (38-40). Supporting the conclusion that both *Dsx*^F and *Dsx*^M are determinants of proper sexual development, either loss of individual *dsx* function or simultaneous gain of both *dsx*^F and *dsx*^M results in an intersexual phenotype (41). Interestingly, despite its crucial role in establishing and maintaining sexually dimorphic differentiation and behavior, *dsx* is expressed in only a select subset of tissues (42, 43). Establishing the biological relevance of the localized expression, inactivation of *dsx* using *dsx-Gal4* resulted in reduction in the size of the ovaries (41). As *dsx* expression was adversely influenced upon ovary specific loss of *Nup107*, we sought to test if *Dsx* plays an important role in female differentiation downstream of *Nup107*.

To assess this possibility, we first tested if compromising *dsx* in the somatic component of the gonad can mimic the *Nup107*^{D364N} phenotypes. Indeed, knockdown of *dsx* expression using somatic driver *tj-Gal4*, resulted in ovarian defects including either partial or complete dysgenesis (in 52% of adult ovaries; Figure. 5A-D). Importantly, compromising *dsx* using *nos-Gal4*, a germline specific driver, did not yield similar phenotypes. Furthermore, *dsx* KD using a *tj-Gal4* driver also led to excess numbers of PGCs as well as a significant number of larval gonads with aberrant IC distribution (Figure. 5E-H). Analysis of germaria compromised for *Dsx*^F function in the ovarian soma largely mimicked the loss of *Nup107* with respect to excess GSCs (distinguishable by the high spherical fusomes; Figure 5I) and loss of the cellular extensions of the ECs (Figure 3E). Importantly, as in the case of *Nup107*, loss of *Dsx*^F in the ovarian soma correlated with aberrant BMP signaling, as reflected in the accumulation of excess nuclear pMad accompanied by the loss of Bam in region 2a of the germaria (Figure. 5J-K).

Figure 5. Knockdown of *dsx* in the gonadal soma recapitulates *Nup107^{D364N}* phenotypes.

(A) *dsx* knockdown in the germline (*nos-Gal4*) results in negligible effects, compared to KD in somatic cells (*tj-Gal4*), which results in both (B) underdeveloped and (C) non-developed ovaries. (D) The percentages of under/non-developed ovaries in *tj-Gal4 dsx* KD (n=74) vs *nos-Gal4 dsx* KD (n=100). (E) Representation of the number of PGCs per confocal section of each individual gonad in *yw* (n=42) and *dsx* KD (n=34) larvae. (F) Larval gonads where *dsx* is knocked down using *tj-Gal4* contain excess PGCs and abnormally dispersed ICs. (G) The percentage of IC severity phenotypes found in *yw* (n=22) and *dsx* KD (n=16) larval gonads. (H) Quantitation of the number of PGCs per gonad lacking an immediately adjacent IC, as a result of abnormal IC dispersion in *yw* (n=22) and *dsx* KD (n=34) larval gonads. (I) *tj*-driven *dsx* KD results in excess spherical fusomes, as well as (J) excess pMad (red) expression and missing Bam (green). (K) Quantitation of *dsx* KD germlaria aberrant phenotypes (n= 53, 119 and 63 respectively).

Dsx overexpression rescues the phenotypes induced by Nup107 loss

The similarity between the *Nup107* mutant and *dsx* KD ovarian phenotypes prompted us to test whether *dsx* is a critical target of *Nup107*. If so, overexpression of *Dsx^F* alone should mitigate the phenotypes induced by the loss of *Nup107*. We therefore knocked-down *Nup107* and concomitantly overexpressed *Dsx^F*. Analysis of the resulting ovarian samples revealed that the proportion of underdeveloped ovaries was substantially diminished as compared to both *Nup107^{D364N}* and *tj-Gal4* driven *Nup107* KD flies (Figure. 6A-C). The gonads rescued upon simultaneous overexpression of *Dsx^F* appeared morphologically normal (Figure. 6B), resembling those of control *yw* females. Furthermore, normal patterns and levels of both pMad and Bam expression were restored in region 2a of the germlaria (Figure. 6D, E). Taken together these data strongly suggested that Dsx is a key component acting downstream of Nup107 and loss of *Dsx^F* could account for the stage and sex specific phenotypes associated with compromised Nup107 activity.

The nearly complete rescue observed by the introduction of *Dsx^F* transgene also implied that the two determinants could share important targets. We tested the notion by comparing the 82 genes, identified as Nup107 targets, with previously reported *Dsx^F* targets (41). Indeed, the comparison revealed that 47 out of the 82 Nup107 targets (57%, $P < 4.5 \times 10^{-11}$) were also identified as targets of Dsx (Table supplement 2). In addition to several transcription factors, included in this list are known modulators of BMP signaling as well as multiple components of the extracellular matrix. The substantial overlap between the two lists thus provides the key to the nearly complete rescue. Importantly these data have begun to elucidate how germline-soma communication engineered by the *Dsx^F* can contribute to the establishment of female germline identity.

Figure 6. Overexpression of Dsx rescues Nup107 KD ovarian phenotypes. (A) RNAi-KD of *Nup107* using *tj-Gal4* driver results in small, underdeveloped ovaries. (B) Co-expression of *dsx* with RNAi-KD of *Nup107* rescues the underdeveloped phenotype, resulting in normal, robust ovaries. (C) Quantitation of under/non-developed ovaries in yw (196), *Nup107* KD (n=316) and *Nup107* KD, *dsx* OE flies (n=250). (D) *tj-Gal4* driven *Nup107* KD, *dsx* OE germarium contains normal pMad (red) expression and (D') normal Bam expression. (E) Quantitation of Bam and pMad expression in yw (n=127, 122), *Nup107* KD (n=64, 48), and *Nup107* KD, *dsx* OE (n=25, 33) germaria. (F) 55% of *tj-Gal4* driven *Nup107* KD ovarioles (n=20) showed apoptosis, marked by anti-Caspase3 (red) compared to (G) zero *Nup107* KD, *dsx* OE ovarioles (n=15).

BMP modulator AdamTS-A acts downstream of Nup107 and Dsx

Our data demonstrate that that *Dsx*^F regulates the range and/or the strength of BMP signaling from the niche. We were thus intrigued by two other candidate genes identified in our differential transcriptome analysis, which have been previously implicated in modulating the range of BMP signaling. These include AdamTS-A, a metalloprotease that controls extracellular matrix (ECM) assembly. Using qPCR

analysis, we found that their expression is appreciably reduced in adult female ovaries compromised for *Nup107* activity as compared to the wild type (Figure. 7G, Figure 7- Figure Supplement 1). Here we have focused our attention on *adamTS-A*, as human ovarian disorders including polycystic ovary syndrome and primary ovarian insufficiency have been correlated with compromised *AdamTS-A* activity (44-46). We first examined if the downregulation of *AdamTS-A*, downstream of *Nup107*, is mediated by *Dsx^F*. Supporting the conclusion that transcription of *adamTS-A* is positively regulated by *Dsx^F*, we observed a significant decrease in the levels of *adamTS-A* transcripts following knockdown of *dsx* (Figure. 7G).

Further, we sought to test if downregulation of *adamTS-A* is an important determinant of ovarian development downstream of *Nup107* and/or *Dsx^F*. Knockdown of *adamTS-A* in the somatic gonadal cells had severe effects on ovarian development including partial ovarian dysgenesis (Figure. 7B, C), increased number of PGCs (Figure. 7D-F) and GSCs (Figure. 7H, I) in the larval and adult female gonads respectively, with expanded levels of pMad and loss of Bam expression as well as loss of ECs' extensions in the germarium (Figures. 7H, J and 3F, respectively). In contrast, germline specific knockdown or disruption of its activity using other tissue-specific drivers (wing, eye etc.) resulted in negligible effects (Figure 7A, C, and data not shown). This finding confirmed *AdamTS-A* as a biologically relevant component that likely acts downstream of both *Nup107* and *Dsx* in this context.

Figure 7. *adamTS-A* KD larval and adult ovaries show aberrant phenotypes. (A) Germline KD of *adamTS-A* (*nos-Gal4*) results in negligible effects, compared to somatic KD (*tj-Gal4*), which results in (B) severely underdeveloped ovaries. (C) Quantitation of under/non-developed ovaries in *tj*- vs *nos-Gal4* driven *adamTS-A* KD (n=126, 108) flies. (D) Somatic KD (*tj-Gal4*) of *adamTS-A* results in larval gonads containing excess numbers of PGCs (VASA, green) and abnormally dispersed Intermingled Cells (TJ, blue). (E) Quantitation of the total number of PGCs per confocal section in each individual gonad in *yw* (n=42) and *adamTS-A* KD (n=17) larvae. (F) The percentage of IC severity phenotypes found in *yw* (n=22) and *adamTS-A* KD (n=17) larval gonads. (G) Relative expression of *adamTS-A* measured by RT-qPCR. (H) Quantification of cells with round fusomes (n=57), Bam expression (n=145, green), and pMad expression (n=73, red) in *yw* and *adamTS-A* KD ovaries. *tj-Gal4* driven *AdamTS-A* KD results in (I) excess number of cells with spherical fusomes (anti-1B1), (J) expanded pMad (red), and (J') reduced Bam expression.

Discussion

We have shown that Nup107 activity in the somatic component of the gonad is necessary for the proper development of the ICs which enables them to interact with the PGCs. Consistent with the notion, loss of Nup107 affected the behavior of ICs such that these cells showed varying degrees of failure to mingle with the PGCs. A severe failure of ICs and PGCs to interact in the larval gonad is expected to cause ovarian dysgenesis-like phenotype as it is essential for the germarium development and ovariole formation. In the adult ovary, loss of Nup107 impaired the differentiation of the ECs, so that they failed to form the cellular extensions necessary for interactions with the GSCs and regulate their differentiation. The adult ovarian ECs are the descendants of the larval ovarian ICs. Collectively, our results are consistent with the model that Nup107 activity is required in the larval gonad for the specification of the ICs, whereas the adult ovarian phenotypes reflect a secondary consequence of compromising Nup107 activity in the progenitors of the ECs. Our data however do not rule out the possibility that Nup107 function is continuously necessary in different cell populations at different times during ovarian development. Future experiments involving selective knockdown of Nup107 in a temporally restricted manner will shed light on the precise function of Nup107 in this context.

Nevertheless, our studies have revealed that Nup107, a ubiquitously expressed nuclear envelope protein, is a crucial player during female gonad formation. How does an essential housekeeping protein critical for nuclear transport, play such a sex and tissue-specific function? We envisage two possible scenarios, not necessarily mutually exclusive, to explain how the specific mutation in Nup107 results in ovary specific aberrant phenotypes. In the first scenario, Nup107 would specifically mediate nucleocytoplasmic translocation of factor(s) or downstream effector(s) required for ovarian development. Indeed, recent studies have demonstrated that Nup107 is involved in translocation of specific factors. For instance, in the event of DNA-damage, Nup107 directly interacts with the apoptotic protease activating factor 1 (Apaf-1 also known as Drosophila ARK) and mediates its transport into the nucleus to elicit cell-cycle arrest (47). Furthermore, it has been shown in tissue culture that specific Nucleoporins, including Nup107, are required for nuclear translocation of SMAD1, an important downstream effector of the Dpp/BMP pathway (48).

Alternatively, accumulating evidence has documented that in addition to their primary function in regulating the exchange of molecules between the nucleus and cytoplasm, NPC components may contribute to genome organization and tissue specific regulation of gene expression (49, 50) in a nuclear transport-independent manner. Consequently, such moonlighting activities may not be confined to the nuclear envelope which is the primary native location of these proteins. For instance, mammalian Nup107-160 complex (a sub-complex of the NPC of which Nup107 is a key component) has recently been shown to shuttle in and out of GLFG nuclear bodies containing Nup98, a nucleoporin that regulates multiple aspects of gene regulation (51). Consistently, Nup107 was shown to regulate levels of specific RNAs through gene imprinting (52). Furthermore, using an RNAi-based assay, Nup107 was identified as a positive regulator of OCT4 and NANOG expression in human ESCs (53). In this regard, it is noteworthy that the recently published chromatin-binding profile of Nup107 suggested that Nup107 specifically targets active genes (54). Altogether these data support the possibility that Nup107 affects transcription of specific target genes in a tissue- and sex- specific manner either directly or indirectly.

In *Drosophila melanogaster*, Sex-lethal (Sxl) is the master determinant of somatic sexual identity, regulating a splicing dependent regulatory cascade resulting in the presence of alternatively spliced sex-specific isoforms of Dsx protein, Dsx-F and Dsx-M, in females and males respectively. Subsequent dimorphic sexual development including sex-specific gonad morphogenesis is under the control of these Dsx isoforms. Consistently, Dsx proteins deploy components of the housekeeping machinery to achieve sex-specific development of the gonads. Thus, such ‘maintenance’ factors are unlikely to be involved in any regulatory capacity. Our data challenge this notion and demonstrate the presence of sexually dimorphic circuitry downstream of a ‘housekeeping’ nuclear envelope protein, Nup107, which regulates the expression the female form of Dsx.

The similar sex-specific and ovary-restricted phenotype associated with compromised Nup107 activity in both humans and flies implies common underlying molecular mechanisms. We have identified Dsx as the primary target acting downstream of Nup107 in *Drosophila* ovarian development. The mammalian homologues of Dsx, the Dmrt family of transcription factors, also function during sex specific gonad development. However, in mammals the main function of *Dmrt* genes in the gonad is to promote male-specific differentiation. While detailed functional

analysis is not available, it is plausible that in mammals, another key female-specific transcription factor, like *Foxl2* (female-specific forkhead box L2) may act downstream of Nup107 to substitute for DsxF in flies.

Our observations have also uncovered that Dsx^F controls somatic niche function by calibrating the range and/or strength of Dpp/BMP signaling, possibly via modulation of the level and/or activity of the extracellular matrix components. Thus, it will be critical to elucidate how activities of non-sex-specific components such as Nup107 are coordinated with sex-specific regulation to achieve the precise specification and patterning underlying gonad development. This is of particular significance since modulation of BMP signaling circuitry is inextricably linked with the establishment and maintenance of stem cell fate. Importantly, as in the case of Nup107, BMP signaling is also required in a non-sex-specific manner in a variety of developmental contexts. These observations therefore open new avenues towards the critical examination of how a productive molecular dialogue is established between non-sex-specific housekeeping machinery and versatile intersecting developmental pathways, in order to ultimately achieve proper sex-specific gonadogenesis crucial for fertility, and transmission of genetic information.

Materials and Methods

Fly Strains.

Flies were raised and maintained at 25°C on standard cornmeal yeast extract media. *yw* was used as a wild-type strain. The generation of *RFP-Nup107^{WT}* and *RFP-Nup107^{D364N}* transgenic flies was previously described in Weinberg-Shukron et al (7). *nanos-Gal4* and *tj-Gal4* were gifted by Lilach Gilboa's lab (Weizmann Institute of Science, IL). *Nup107* RNAi and *adamTS-A* RNAi lines were provided by the Vienna Drosophila Research Center (VDRC, Vienna, Austria #108047 and #110157). *dsx* RNAi and *dsx* UAS provided by Bloomington Drosophila Stock Center (BDSC; Indiana University; USA; #35645, #41864 and #44223).

The lines' complete genotypes:

1. *yw*: *y¹w^{*}* ; ;
2. *RFP-Nup107^{WT}*: *y¹w^{*}* ; *Nup107^{E8}/Nup107^{E8}*; *RFP-Nup107^{WT}*
3. *RFP-Nup107^{D364N}*: *y¹w^{*}* ; *Nup107^{E8}/Nup107^{E8}*; *RFP-Nup107^{D364N}*
4. *nanos-Gal4*: *w^{*}* ; ; *nos-GAL4::VP16*
5. *tj-Gal4*: *w^{*}* ; *tj-Gal4/ Cyo*;
6. *Nup107* RNAi: *y¹w^{*}* ; *UAS-Nup107-RNAi*;
7. *adamTS-A* RNAi: *w^{*}* ; *UAS- adamTS-A RNAi*;
8. *dsx* RNAi: *y¹*; *UAS- dsx -RNAi/Cyo*; or *y¹* ; ; *UAS- dsx -RNAi*
9. *UAS dsx*: *y¹w^{*}* ; ; *UAS- dsx*;

The crosses genotype:

1. *tj-Gal4>Nup107* RNAi: ; *tj-Gal4/ UAS-Nup107-RNAi*;
2. *nanos-Gal4>Nup107* RNAi: ; *UAS-Nup107-RNAi*; *nos-GAL4::VP16*
3. *tj-Gal4>adamTS-A* RNAi: ; *tj-Gal4/ UAS- adamTS-A RNAi*;
4. *tj-Gal4>dsx* RNAi: ; *tj-Gal4/ UAS- dsx -RNAi*; or *w*; *tj-Gal4; UAS- dsx -RNAi*
5. *tj-Gal4>Nup107* RNAi, *UAS dsx*: ; *tj-Gal4/ UAS-Nup107-RNAi*; *UAS dsx*

Adult and Larval Gonad Dissections.

Stage LL3 larvae were collected and subsequently dissected for their gonads according to Gilboa and Maimon (55). Adult ovaries were dissected from 3-5 day old females placed on yeast for 24-36 hours in the company of males. All experiments

were performed at 25°C, and all were independently repeated at least twice. Dissection was performed in Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 50 mM Na₂HPO₄, pH 7).

Immunostaining and imaging.

Fixation and immunostaining of larval gonads or adult ovaries were carried as previously described by Maimon (55) or by Preall (56). In brief, gonads or ovaries were fixed in freshly prepared 5% paraformaldehyde (PFA; Electron Microscopy Sciences; Cat# 15714) for 30 min at room temperature. Blocking was carried out in wash buffer supplemented with 1% bovine serum albumin (BSA; MP Biomedicals; Cat. #160069). Primary antibodies were diluted and incubated overnight at 4°C in wash buffer supplemented with 0.3 % BSA. The following primary antibodies were used: guinea-pig anti-Tj (1:10,000; gifted by Dorothea Godt's lab at the University of Toronto, Toronto, CA), rat IgM anti-VASA (1:100; Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA)), mouse anti-Hts (1B1; 1:20; DSHB), mouse anti-Bam (1:50; DSHB), mouse anti-Cora (C566.9; 1:100; DSHB), rabbit anti-smad3 (1:100; abcam, Cambridge, MA, USA; #ab52903), rabbit anti-cleaved caspase3 (1:200; Cell Signaling Technology, CST, Danvers, MA, USA; #9661) and mouse anti Lamin C (1:20; gifted by Yosef Gruenbaum's lab at the Hebrew University of Jerusalem, IL). Secondary antibodies (1:400) were conjugated to either Cy2, Cy3 or Cy5 (Jackson Immuno Research Laboratories; West Grove, PA, USA). Ovaries were mounted in Vectashield (Vector Laboratories; Burlingame, CA, USAVE-H-1000). Images were taken on a TE2000-E confocal microscope (Nikon) using x20 or x60 objectives, occasionally with an additional x1.5 zoom. Figures were edited using Adobe Photoshop CC 2017.

Quantitative real-time PCR analysis.

Total RNA was isolated from larval gonads at stage LL3 (~80 per sample) of *yw*, *Nup107^{WT}* and *Nup107^{D364N}* using the RNeasy mini kit (Qiagen Valencia, CA, USA; #74104). Briefly, wandering third instar larvae were collected, females were selected and dissected into tubes in liquid nitrogen and lysis buffer was added. Total RNA was extracted as per the manufacturer's instructions. cDNA was made using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) using an equal amount of total RNA from each sample. Real-time q-PCR

analyses were carried out using the Powersyber Green PCR Master Mix and QuantStudio 12k flex (Applied Biosystems). Rsp17 and TBP2 served as reference genes using the comparative Ct method. Each sample was analyzed in triplicate; results were confirmed by at least two independent experiments. Primer sequences (from HyLabs, Israel, LTD) used for qPCR were:

dm_Doublesex_F, 5'-TTGCCGATCTCAGTTTCCGT-3';
 dm_Doublesex_R, 5'- GCTCCCAAGGATAGCGGAAT-3';
 dm_AdamTS-A_F, 5'- GGGAATGAGCCGAACAAGAC-3';
 dm_AdamTS-A_R, 5'- AAGTTCTGGTCGGGATAGCC-3'.

Statistical Analysis

The number of under/non-developed adult ovaries in wild-type and mutant flies, the varied expression of 1B1, bam, Cora, as well as the number of spherical fusomes in germaria were compared pairwise using Fisher's Exact Test for 2x2 tables. The raw 2-tail p-values were adjusted for the multiple comparisons using either the Bonferroni correction or Holm's modification (57) thereof, as appropriate. P values of less than 0.05 were considered significant.

The number of Primordial Germ Cells, Intermingled Cells and fusomes in groups of larval gonads were compared using a Kruskal-Wallis Test (58). In experiments where the differences among the groups were found to be significant (K-W p-value <0.05), pairwise comparisons were carried out using Conover's post-hoc test (59).

The statistical significance of the observed overlap between our Nup107 target genes list and the previously reported Dsx targets was calculated using the hypergeometric test (<https://systems.crumph.ucla.edu/hypergeometric/index.php>). The specific parameters were as follows: number of successes k=47; sample size s=82; number of successes in the population M=3717; population size N=15835. The results obtained were: expected number of successes = 19.2481212503947. The results are enriched 2.44 fold compared to expectations hypergeometric p-value = 4.4742336544062e-11.

RNA-Seq

Total RNA was isolated from *yw*, *RFP-Nup107^{WT}*, and *RFP-Nup107^{D364N}* *Drosophila* larval gonads following dissection at stage LL3 using the RNeasy Kit (Qiagen) according to the manufacturer's protocol. RNA purity and concentration were determined by T-042 NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and integrity by 2100 Bio-analyzer (Agilent Technologies, CA, USA). Total RNA was reverse transcribed to cDNA using SENSE Total RNA-Seq Library Prep Kit for Illumina (Lexogen, Vienna, Austria), according to the manufacturer's protocol, with poly-A selection. Libraries were multiplexed and sequenced on Illumina's NextSeq 500 machine, with a configuration of 75 cycles, single read. Raw reads were processed to remove low quality, error prone and adapter sequences, according to Lexogen's SENSE libraries recommendations. High quality reads were aligned to the fly genome, assembly BDGP6, that was supplemented with the sequences of GFP and the RFP-Nup constructs. Alignment was performed with TopHat, allowing for up to 5 mismatches per read. Differential expression analysis, for all genes from release 84 of the Ensembl database, was performed with the DESeq2 package, using default parameters, including the threshold for significance that was $\text{padj} < 0.1$. Significant genes were further filtered to include only genes whose up- or down-regulation was greater in the *mut/yw* comparison than in the *wt/yw* one. For that end, a difference of at least 0.1 between the absolute $\log_2\text{FoldChange}(\text{mut/yw})$ and absolute $\log_2\text{FoldChange}(\text{wt/yw})$ was used as the filtering threshold. All raw data, as well as software versions and parameters, have been deposited in NCBI's Gene Expression Omnibus (60) and are accessible through GEO Series accession number GSE141094.

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Abbreviations used in this paper: AdamTS-A, A disintegrin-like and metalloprotease domain [reprolysin type] with thrombospondin type 1 motifs; *bam*,

bag of marbles; BMP, Bone Morphogenetic Protein; Cora, Coracle; Dpp, Decapenataplegic; Dsx, Doublesex; ECM, extracellular matrix; ECs, Escort cells; *Foxl2*, female-specific forkhead box L2; GSC, germline stem cells; ICs, Intermingled Cells; KD, knockdown (KD) ; LL3, late third instar larval stage; *Nup107*, nucleoporin-107; *nos-Gal4*, *nanos-Gal4*; PGCs, primordial germ cells; pMad, phosphorylated Mothers against dpp; TF, terminal filament; Sxl, Sex-lethal; Tkv, Thickveins; *tj-Gal4*, *traffic jam-Gal4*; XX-OD, XX ovarian dysgenesis.

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Author contributions: T.S. and T.L. designed, performed, and analyzed the majority of the experiments, with R.K., A.D, and D.R. being involved in the investigation and analysis at earlier stages; and M.Y.G. and S.L. being involved in the later stages. T.S. performed all gonadal analyses while T.L. performed germarium experiments. A.W.S. performed the early stages of the molecular work. Experiments in the wing and eye discs were carried out by T.B. and V.M. T.S. and D.R. extracted the gonadal tissue for the RNA-Seq. RNA-Seq bioinformatic analysis was performed by Y.N. Funding was acquired by O.G., D.Z., and G.D. The project was conceptualized by O.G and G.D. The original manuscript was written by T.S., O.G. and G.D., and subsequently reviewed and edited by T.L and M.Y.G., O.G. supervised the project.

Competing interests: The authors declare no competing interests.

Data and materials availability: All raw RNA-seq data, as well as software versions and parameters, have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE141094. All other data is available in the main text or the supplementary materials.

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Supplementary Files

Supplementary Figure legends

Figure 3-Figure supplement 1. Female larval gonads compromised for *Nup107*

show characteristic aberrations. (A) Unlike their adult counterparts, the *Nup107*^{D364N} female larval gonads are present at LL3. **(B)** The difference between the total number of cells with spherical fusomes in *yw* (n=11) *Nup107*^{WT} (n=13) and *Nup107*^{D364N} (n=17) larval gonads is significant. **(C)** Similar numbers of cells with round fusomes (anti-1B1) can be seen in *yw* and *Nup107*^{WT} gonads, while being substantially elevated in **(D)** *Nup107*^{D364N} gonads.

Figure 4-Figure supplement 1. Total number of Intermingled Cells (ICs) remains

unaffected in the larval gonads compromised for *Nup107*. (A) The difference between the average number of ICs in *yw* (n=13), *Nup107*^{WT} (n=14) and *Nup107*^{D364N} (n=12) larval gonads is statistically insignificant. P value represents comparison of all three groups.

Figure 4-Figure supplement 2. Reduction in *dsx* and *adamTS-A* transcription

upon compromising *Nup107* activity in the *Drosophila* larval gonad. (A) Quantitative real-time PCR based validation was performed by normalizing the relative expression levels to the abundance of housekeeping gene RpS17.

Figure 7-Figure supplement 1. Collagen type IV alpha 1 (*col4a1*) expression levels

are reduced in both *Nup107* KD and *dsx* KD adult ovaries. (A) Relative expression of *col4a1* in the *Drosophila* adult ovary, measured by RT-qPCR. RpS17 and TBP2 expression were used as controls. Statistical significance was calculated using the comparative Ct method.

Supplementary tables

Table supplement 1. Processed data results of transcriptomic analysis performed on *Nup107*^{WT} and *Nup107*^{D364N} larval gonads.

Table supplement 2. List of 82 candidate genes with differential expression following *Nup107* loss identified in transcriptomic analysis, with emphasis on those found to be *dsx* targets.