

# The Pygo2-H3K4me2/3 interaction is dispensable for mouse development and Wnt signaling-dependent transcription

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## SUMMARY

Pygopus has been discovered as a fundamental Wnt signaling component in *Drosophila*. The mouse genome encodes two Pygopus homologs, Pygo1 and Pygo2. They serve as context-dependent  $\beta$ -catenin coactivators, with Pygo2 playing the more important role. All Pygo proteins share a highly conserved plant homology domain (PHD) that allows them to bind di- and trimethylated lysine 4 of histone H3 (H3K4me2/3). Despite the structural conservation of this domain, the relevance of histone binding for the role of Pygo2 as a Wnt signaling component and as a reader of chromatin modifications remains speculative. Here we generate a knock-in mouse line, homozygous for a Pygo2 mutant defective in chromatin binding. We show that even in the absence of the potentially redundant Pygo1, Pygo2 does not require the H3K4me2/3 binding activity to sustain its function during mouse development. Indeed, during tissue homeostasis, Wnt/ $\beta$ -catenin-dependent transcription is largely unaffected. However, the Pygo2-chromatin interaction is relevant in testes, where, importantly, Pygo2 binds *in vivo* to the chromatin in a PHD-dependent manner. Its presence on regulatory regions does not affect the transcription of nearby genes; rather, it is important for the recruitment of the histone acetyltransferase Gcn5 to chromatin, consistent with a testis-specific and Wnt-unrelated role for Pygo2 as a chromatin remodeler.

**KEY WORDS:** Wnt signaling, Chromatin, Transcriptional regulation, Pygopus, Mouse, *Drosophila*

## INTRODUCTION

Pygopus was first identified as an essential, dedicated Wg/Wnt signaling component in *Drosophila melanogaster* (Kramps et al., 2002; Thompson et al., 2002; Parker et al., 2002) and in *Xenopus* (Belenkaya et al., 2002). Epistatic and molecular analyses showed that Pygopus acts downstream of Arm/ $\beta$ -catenin to efficiently activate Wg/Wnt target gene transcription (Mosimann et al., 2009). Most likely this is achieved by Pygopus being tethered to the  $\beta$ -catenin transcriptional complex via the protein Lgs/Bcl9, and thus contributing to the activation of Wnt-target gene transcription via the conserved N-terminal homology domain (NHD) (Hoffmans et al., 2005). Pygopus binding to Lgs/Bcl9 is mediated by a few crucial amino acids located within the evolutionarily conserved plant homology domain (PHD), and in *Drosophila* this interaction is indispensable for development. However, other mechanisms have been proposed, including one in which Pygo constantly associates with Wnt target loci and contributes to the nuclear import of  $\beta$ -catenin upon signal induction (Townsend et al., 2004b; de la Roche and Bienz, 2007).

In mouse, two Pygopus genes are present, the paralogs *Pygo1* and *Pygo2*, with *Pygo2* being more widely expressed (Li et al., 2004). Surprisingly, knockout mutants of Pygo1/2 proteins produce phenotypes considerably less severe than the axial patterning defects in early embryogenesis observed in mice null mutant for  $\beta$ -catenin (Haegel et al., 1995) or expressing only a transcriptionally inactive mutant of  $\beta$ -catenin (Valenta et al., 2011). More specifically, *Pygo1* null mice are viable and fertile, whereas mice deficient for *Pygo2* die shortly after birth, presenting a series of developmental defects:

encephaly, decreased hair follicle density, aberrant lung morphology, reduced or absent eye lens, and aberrant branching morphogenesis during kidney development (Li et al., 2007; Schwab et al., 2007). Surprisingly, mice deficient for both *Pygo1* and *Pygo2* do not display an exacerbated phenotype, suggesting that Pygo2 is playing the more important role (Schwab et al., 2007). In the adult, decreased Pygo2 expression causes a defect during spermiogenesis, due to a putative acetylation-dependent structural role of Pygo2 in chromatin condensation during terminal differentiation of sperm cells (Nair et al., 2008). Although some of these defects are attributable to reduced Wnt signaling, Pygo2 has also been reported to act in a Wnt/ $\beta$ -catenin independent fashion in lens morphogenesis and spermiogenesis (Song et al., 2007; Nair et al., 2008).

In its C-terminus Pygo proteins have a PHD, a highly structured Zn<sup>2+</sup> coordinating finger (Nakamura et al., 2007). Several studies have shown that the PHD-containing proteins can act as ‘code readers’, linking chromatin remodeling to changes in gene transcription (Li et al., 2006; Peña et al., 2006). The PHD can bind di- and trimethylated lysine 4 on histone H3 (H3K4me2/3). The mammalian Pygo-PHD is no exception and is able to bind H3K4me2/3 *in vitro* (Kessler et al., 2009; Fiedler et al., 2008). However, it is debated whether the Pygo-H3K4me2/3 association has a functional relevance. In *Drosophila*, the Pygo-PHD domain carries an altered residue in a highly conserved region, and is thus unable to bind H3K4me2/3. Moreover, transgenes in which the PHD domain is deleted can efficiently rescue *pygo* mutant flies if the Pygo-NHD is covalently bound to Lgs (Kessler et al., 2009). Data obtained in mice, however, imply that PHD-mediated Pygo2 chromatin binding supports and enhances mammary gland progenitor proliferation by facilitating H3K4 methylation (Gu et al., 2009). Thus *Drosophila melanogaster* may represent an evolutionary exception, whereas in other organisms Pygo chromatin binding may play a more important role.

Here we set out to directly test this hypothesis *in vivo* and engineered a mouse *Pygo2* knock-in allele bearing a mutation within the PHD that essentially abolishes its H3K4me2/3 binding

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activity. We find that Pygo2 normally binds chromatin *in vitro* and *in vivo*, but that this function is largely dispensable during mouse development and tissue homeostasis. Indeed, even in the absence of Pygo1, this abrogation does not affect Wnt signaling-related transcription. Of note, compromised Pygo2-chromatin binding leads to reduced male fertility, owing to a defect in the production of mature sperm cells.

## MATERIALS AND METHODS

### Generation of Pygo2 knock-in mouse strain

A knock-in mutant of the *Pygo2* locus was generated by standard techniques (inGenious Targeting Laboratory, USA). Briefly, the targeting vector was generated and electroporated into BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem (ES) cells. After selection with the antibiotic G418, surviving clones were expanded for PCR and Southern blotting analyses to confirm recombinant ES clones, followed by validation of the point mutation. mESCs harboring the knock-in allele were microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to wild-type C57BL/6N mice to generate F1 heterozygous offspring. Neo-cassette excision in somatic cells was obtained, as schematized in Fig. 1B, by crossing heterozygous knock-in animals with mice expressing Flp-recombinase under the control of cytomegalovirus promoter. Further details are available upon request.

### Mouse experiments and genotyping

Mouse experiments were performed in accordance with Swiss guidelines and approved by the Veterinarian Office of the Kanton of Zurich, Switzerland. Genotyping of the *Pygo2-A342E* allele was performed using sequence-specific primers that allow the detection of the remaining flippase recognition target (FRT) site after the Flp-mediated Cre-cassette excision (Fig. 1B,C). *LoxP*-containing *Pygo1* alleles were generated according to the scheme shown in supplementary material Fig. S2; a knockout mutant allele was obtained by crossings with Nestin-*Cre* transgenic mice. Primers were designed spanning the *Pygo1 LoxP*-flanked region. As control, a set of primers spanning a single *LoxP* site was used to detect the *LoxP*-containing *Pygo1* allele before the recombination. To monitor Wnt/ $\beta$ -catenin transcription, knock-in animals were crossed with the *BAT-gal* reporter line (Maretto et al., 2003). The presence of the *BAT-gal* reporter has been confirmed using primers amplifying  $\beta$ -galactosidase coding sequence.

### Quantitative RT-PCR

Total RNA was extracted from mouse testes, kidneys, embryonic liver and mouse embryonic fibroblasts (MEFs) using TRIzol (Invitrogen). Quantitative real-time SYBR-Green-based PCR reactions were performed in triplicate and monitored with the ABI Prism 7900HT system (Applied Biosystems). Specific primers were designed to amplify 70–150 bp intron-spanning coding regions, and were based on sequences from the Ensembl database ([http://www.ensembl.org/Mus\\_musculus/Info/Index](http://www.ensembl.org/Mus_musculus/Info/Index)). Dissociation curves confirmed the homogeneity of PCR products. Samples from three or more independent animals were analyzed, each in triplicate.

### Western blot

Total and nuclear protein extracts were prepared according to standard protocols, and proteins were subjected to SDS-PAGE separation and blotting. The following antibodies were used: anti-Pygo2 (Novus Biological, NBP1-46171, and Santa Cruz, sc-74878); anti-histone H3 (Abcam, ab1791); anti-H3K4me3 (Abcam, ab8580); anti-acetyl-K9/K14 histone H3 (Millipore, 06-599); anti-acetyl-K56 histone H3 (Epitomics, 2134-1); anti-pan-acetyl histone H4 (Active Motif, 39244); anti-tubulin (Sigma); anti-E-cadherin (Santa Cruz, sc-7870). Antibody binding was detected by using an appropriate horseradish peroxidase-conjugated IgG and revealed by ECL (GE Healthcare).

### Chromatin immunoprecipitation

Briefly, testes and kidneys were rinsed and mechanically dissociated, fixed with 1% formaldehyde for 10 minutes at room temperature, and lysed. Chromatin was sonicated to a size between 200 and 1000 bp. Immunoprecipitation was performed after overnight incubation with

specific antibodies, anti-Pygo2 (Novus Biological, NBP1-46171, and Santa Cruz, sc-74878) and anti-Wnt3 (Santa Cruz, SC-28824), followed by addition of protein A agarose beads (Upstate Biotechnology). Immunoprecipitated DNA was then analyzed by quantitative PCR (see quantitative RT-PCR section). For each locus tested two primer sets were designed: one detecting a region close to the transcriptional start site, and another 1 kb upstream. All the primer sequences are available upon request. Each immunoprecipitation reaction was repeated three times on independent chromatin preparations.

### *In vitro* pull down

Kidneys and testes were minced in cold PBS, and derived cells treated with a hypotonic lysis buffer (20 mM Tris-HCl, 75 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% NP40, 5% glycerol). Protein extracts obtained were incubated with 5  $\mu$ g biotinylated-H3K4me2 peptide (1-21 amino acids, Upstate) and Streptavidin-conjugated Sepharose beads (GE Healthcare); they were then diluted in lysis buffer to a final volume of 1 ml. After 4 hours of incubation at 4°C on a rotating wheel, the beads were spun down, and washed three times in lysis buffer. All steps were performed on ice, and all buffers were supplemented with fresh protease inhibitors (Complete, Roche) and 1 mM PMSF. The beads were then treated with Laemmli buffer, boiled at 85°C for 15 minutes. Immunoblotting was performed to detect the presence of Pygo2.

### Immunohistochemistry

Cryosections were fixed for 15 minutes in 4% paraformaldehyde in PBS at room temperature, blocked with 10% goat serum, 0.3% Triton X-100 in PBS, and incubated over night at 4°C with primary antibody (mouse anti- $\beta$ -galactosidase, Promega, 1:2000). Slides were washed three times with blocking buffer, and then incubated with a fluorescently labeled secondary antibody (Alexa Fluor 488 goat anti-mouse, 1:400). Nuclei were stained with DAPI (1:1000, Sigma).

## RESULTS

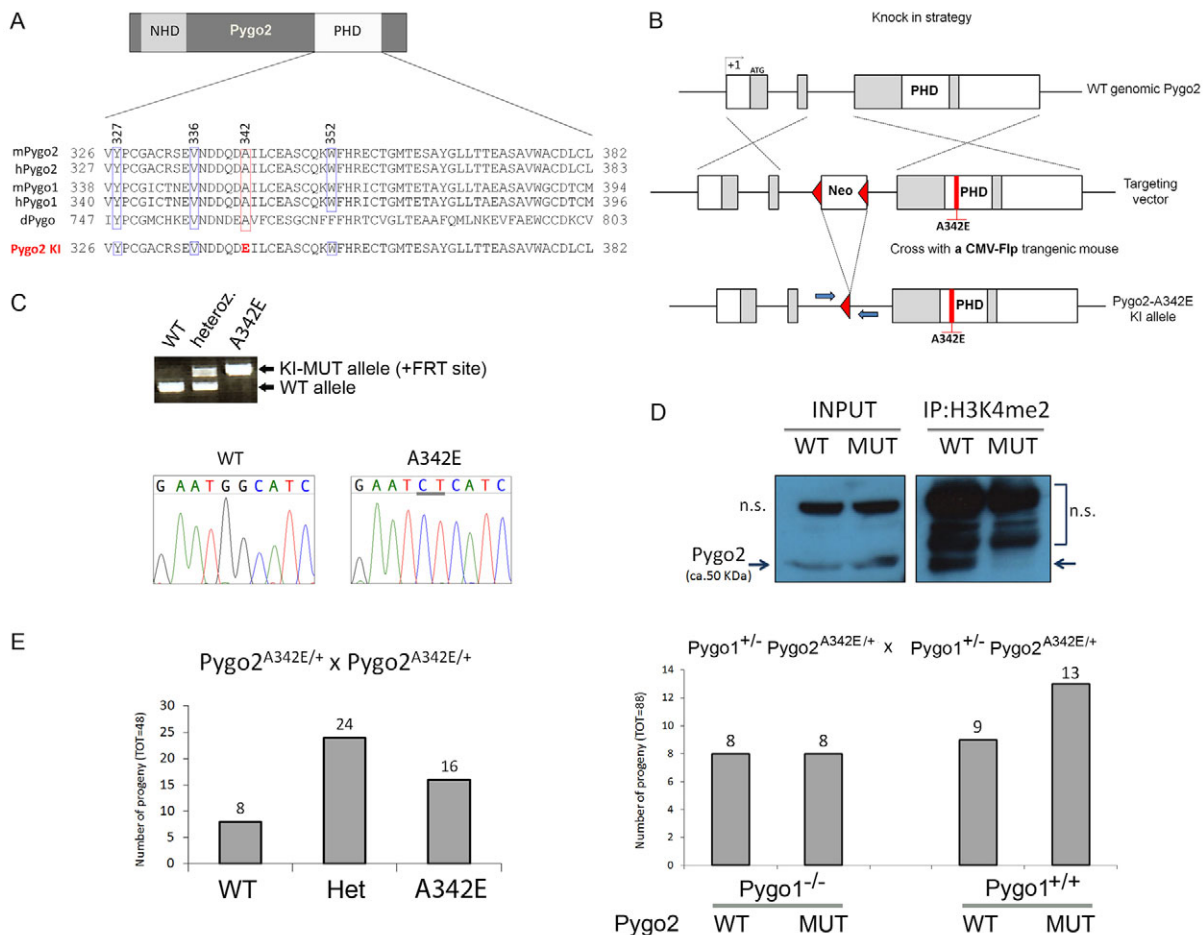
### Generation of a knock-in allele coding for a Pygo2 defective in chromatin binding

In order to study the biological relevance of the Pygo-H3K4me2/3 interaction in the mouse, we generated a *Pygo2* knock-in mutant allele, bearing a two-nucleotide change (CC>AG) within the PHD, which leads to a substitution of the alanine residue in position 342 with a glutamic acid (A342E mutation; Fig. 1A,B). This residue is part of a flexible surface loop that binds the K4 pocket of histone H3; the binding of Pygo to H3K4me2/3 is effectively abolished by this change – the reduction in binding affinity is at least 50-fold (Fiedler et al., 2008; Kessler et al., 2009).

### The ability of Pygo2 to bind H3K4me2/3 is not necessary for mouse development

Once we had generated heterozygous mice carrying one copy of the *Pygo2-A342E* allele, we set up timed breedings to obtain homozygous embryos. Genotyping was performed using sequence-specific PCR primers, followed by DNA sequencing to confirm the presence of the A342E mutation (Fig. 1B,C). Analyses at different stages during embryonic development allowed us to exclude any obvious developmental defect in those organs and tissues in which an impairment due to Pygo2 absence has been previously described (E13.5 in supplementary material Fig. S1; E15.5 and E18.5 not shown) (Li et al., 2007). At all stages, homozygous mutant mice were present in Mendelian ratios (not shown). Of great surprise, homozygous animals were even born in Mendelian ratios (Fig. 1E, left panel), without displaying any obvious abnormality, and reached adulthood along with wild-type littermates.

The abrogating effect of the mutation on the Pygo-H3K4me2/3 interaction was confirmed by *in vitro* pull-down experiments using streptavidin-conjugated Sepharose beads of a biotinylated



**Fig. 1. Generation of a histone-binding defective *Pygo2* knock-in allele.** (A) Degree of conservation of Pygo PHD domains among human, mouse and *Drosophila*. Amino acid residues constituting the 'K4 cage' are indicated with blue squares (Fiedler et al., 2008). The targeted residue, and the corresponding substitution in the knock-in allele, is indicated in red. Position numbers indicated above are relative to the mPygo2 sequence. (B) Targeting knock-in vector and strategy for replacing the mouse *Pygo2* locus with the *Pygo2*-A342E allele. Recombination is followed by the Flp-mediated Neo-cassette excision. Blue arrows indicate the position of the primers used for PCR-based genotyping. (C) PCR-mediated genotyping detects the remaining FRT site, producing from the knock-in allele a higher band than the one obtained with wild-type genomic DNA. Sequencing of a region within the PHD confirms the presence of the mutation. (D) Pull down of a biotinylated H3K4me2 peptide with streptavidin-coated Sepharose beads, followed by immunoblotting with a Pygo2 antibody. Nonspecific signals (n.s.) obtained when using Pygo2 antibody are used as loading control. (E) Genotype ratios obtained from crossings of *Pygo2*<sup>A342E/+</sup> heterozygous (left) or from *Pygo1*<sup>+/-</sup>;*Pygo2*<sup>A342E/+</sup> double heterozygous mice (right). KI, knock-in.

H3K4me2 peptide, incubated with protein extracts derived from kidney, the organs in which Pygo2 displays the highest expression (Li et al., 2004). Although the H3K4me2 peptide was able to pull down Pygo2 from a wild-type extract with high efficiency, no Pygo2 protein was detected in the knock-in lane (Fig. 1D). This result validates the mutation and further confirms that, at least *in vitro*, Pygo proteins are indeed able to bind the methylated histones. Of note, despite a previous report, which implicated the histone-binding ability of Pygo2 in the proliferation of mammary gland progenitor cells (Gu et al., 2009), *Pygo2*-A342E homozygous females can mate and properly feed their progeny (a more detailed analysis of the mammary gland tissue is currently underway).

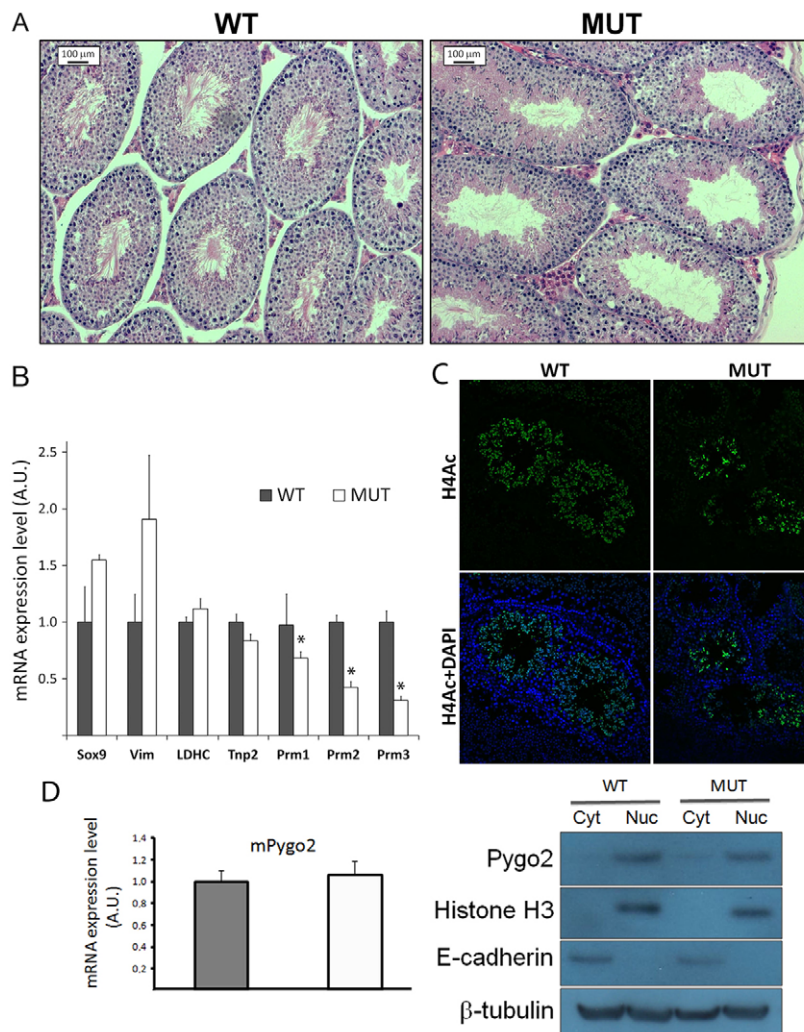
It has been shown that Pygo1 cannot compensate for the complete loss of Pygo2 (Schwab et al., 2007). Moreover, we found that *Pygo1* expression remains unchanged in homozygous *Pygo2*-A342E tissues (data not shown). Despite this, we felt it was important to completely exclude the possibility that Pygo1 is functionally redundant with Pygo2. We transferred the *Pygo2*-A342E mutation into a *Pygo1*<sup>-/-</sup> background (a conditional *Pygo1* knockout allele

was generated according to the scheme shown in supplementary material Fig. S2). *Pygo1*<sup>-/-</sup>; *Pygo2*<sup>A342E/A342E</sup> mice (hereafter referred to as MUT) were born in the expected Mendelian ratios (Fig. 1E, right panel), and were indistinguishable from their wild-type littermates. We conclude that our genetic approach, the single amino acid substitution in Pygo2, combined with the complete deletion of *Pygo1*, conclusively demonstrates that the ability of Pygo proteins to bind H3K4me2/3 is functionally dispensable during mouse development.

### Mutant male mice display an impaired fertility

Despite being otherwise normal in appearance and behavior, ~50% of the MUT male mice are sterile. The rest have a reduced fecundity (they need to be left longer in breeding to give rise to descendants). The mutation does not, however, affect the breeding behavior, and all MUT males copulate and leave a vaginal plug.

The observed impaired fertility is consistent with the recently reported role of Pygo2 during spermiogenesis, in which decreased levels of nuclear Pygo2 led to dysfunctional histone acetylation and



**Fig. 2. Testis-specific defect of Pygo2 MUT male mice.** (A) Magnification (25 $\times$ ) of sections from wild-type (left panel) and MUT (right panel) testes. Several seminiferous tubules devoid of mature sperm cells are visible in the MUT animals. Elongating spermatids in the wild type are often mirrored by cells displaying less condensed and not elongated nuclei. (B) Quantitative RT-PCR showing the expression of selected testis-specific genes. The data represent the average of gene expression from three different MUT mice. The expression of each gene in the wild type has been set to 1. A statistical significance of  $P < 0.05$  is indicated by asterisks. (C) Sections of seminiferous tubules were stained with an antibody against acetylated histone H4 (H4Ac, green). DAPI (blue) stains the nuclei. (D) The histone-binding mutation does not affect *Pygo2* mRNA expression and stability (quantitative RT-PCR, left panel), nor its nucleus/cytoplasm distribution (western blot, right panel). A.U., arbitrary units; WT, wild type.

a block in sperm cell production (Nair et al., 2008). To test whether abolishing the chromatin-binding ability of Pygo2 leads to a similar phenotype we studied in the MUT testis: (1) the seminiferous tubules, (2) the expression of testis-specific genes, and (3) the level of histone acetylation. Testicle sections display a severe depletion of mature sperm cells in the lumen of most of the seminiferous tubules in MUT animals, with impaired nuclear condensation of elongating spermatids, similar to observations by Nair et al. (Nair et al., 2008) (supplementary material Fig. S3; Fig. 2A, upper panels). We also saw fewer condensed nuclei in the MUT seminiferous tubules by propidium iodide and DAPI staining (not shown).

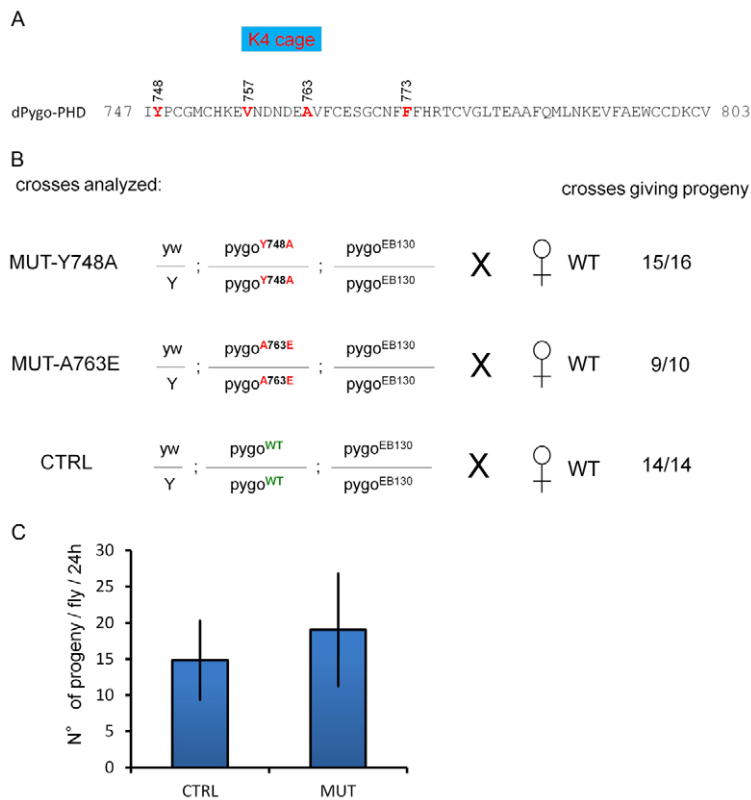
By quantitative RT-PCR we detected a decreased expression of postmeiotic markers, such as the testis-specific histone variants *Prm1*, *Prm2* and *Prm3*; expression of premeiotic markers such as *Ldhc* was unaffected (Fig. 2B). Interestingly, several mice displayed a statistically nonsignificant upregulation of *Sox9* and *Vim*, two markers of Sertoli cells. This might indicate that in MUT testis the proportion of cells at different stages of maturation is changed, possibly because of a partial loss of terminally maturing spermatids. Proliferation and apoptosis are not affected in the MUT testicles, as shown by staining of testis sections with antibodies against phosphohistone H3 and cleaved caspase 3, respectively (supplementary material Fig. S4), supporting the notion that an impairment in sperm cell differentiation (rather than the inability of progenitors to proliferate, or their premature loss) is the reason of

the observed sterility. Finally, we detected an attenuation in the acetylation levels of lysines on histones H3 and H4 in cryosections of MUT when compared with wild type (Fig. 2C).

The testis-specific defect described by Nair et al. (Nair et al., 2008) has been shown to be causally linked to an alteration of Pygo2 expression and nuclear/cytoplasmic distribution. Owing to the phenotypic resemblance to our findings, we wanted to test whether the Pygo2-A342E mutation also causes such aberrant subcellular localization. We monitored Pygo2 expression, by measuring the mRNA and protein, and its subcellular distribution, via a nucleus/cytoplasm fractionation of whole testis cells. We found that both parameters were unaffected in the MUT versus wild-type testes (Fig. 2D). As expected, Pygo2, irrespective of whether wild type or mutant, was predominantly found in the nucleus (Fig. 2D, right panel). Taken together, these observations highlight the role of Pygo2 during the last phases of sperm cell differentiation and corroborate the link between Pygo2 and histone acetyltransferase activity. Importantly, the results indicate that to execute these roles the ability of Pygo2 to bind H3K4me2/3 is needed.

### The Pygo role in male fertility is not conserved in *Drosophila melanogaster*

The inherent histone-binding ability of *Drosophila* Pygo (dPygo) is negligible when compared with the mouse and human Pygo proteins, owing to the presence of a phenylalanine (F) in place of



a tryptophan (W) within the K4 cage of the PHD (position 773, Fig. 1A; Fig. 3A). Moreover, a histone-binding defective dPygo transgene proved to be as efficient as a wild-type transgene in rescuing *pygo* mutant flies (Kessler et al., 2009). Nevertheless, there is still H3K4-binding potential and dPygo might play a role in male sperm cells, in which binding to methylated histones is required. Indeed, an incompletely penetrant fertility defect, as we observe in mice, may also be present in *Drosophila*, but could have gone unnoticed because male flies are usually mated in groups to females, with space and food representing the only limiting resources. We therefore tested if flies expressing only a non-histone-binding variant of dPygo (dPygo-Y748A or dPygo-A763E – the latter is analogous to the A342E mutation in mice, in a *pygo*<sup>EB130</sup> mutant background) exhibit reduced fecundity (Fig. 3B). We set up crosses of single males with three females per tube, and counted the number of males capable of having progeny, as well as the number of viable progeny per female obtained in a 24-hour time period. Almost all the mutant males were able to give rise to viable progeny (Fig. 3B, right column), and no statistically significant difference was scored in the number of F1 progeny (Fig. 3C). Thus, in contrast to the situation in mice, abrogating the ability of Pygo to bind histones has no phenotypic consequences in *Drosophila*.

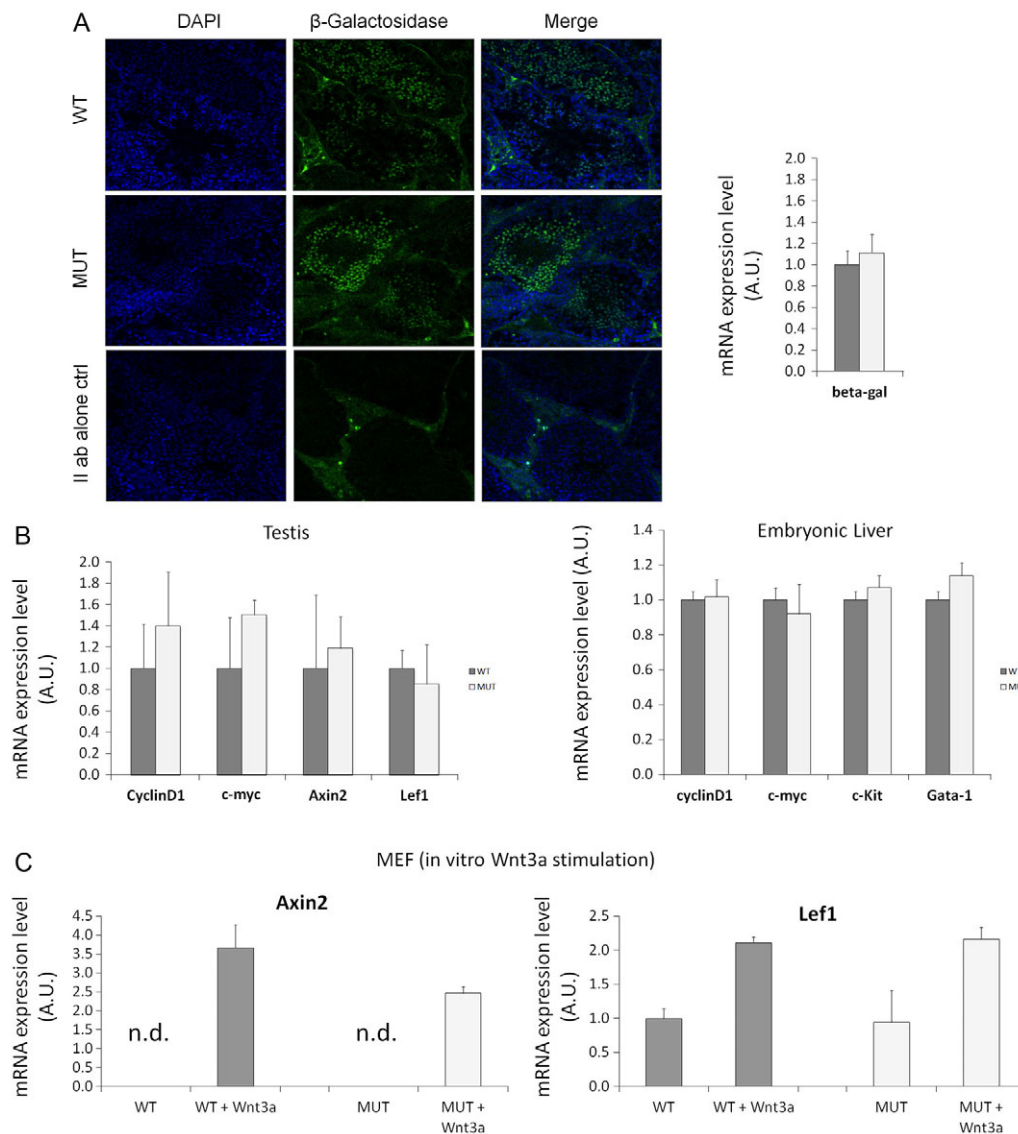
### The Pygo2-A342E mutation does not affect canonical Wnt signaling

dPygo has been reported to be an essential and dedicated Wg/Wnt signaling component, acting as a Arm/β-catenin transcriptional cofactor (Kramps et al., 2002; Hoffmans et al., 2005; Carrera et al., 2008). It can also bind together with Pan/Tcf to target loci in the absence of pathway activity, and may facilitate the recruitment of Arm/β-catenin via the adapter protein Lgs/Bcl9 in the presence of extracellular Wg ligand (Townesley et al., 2004a; Townesley et al.,

**Fig. 3. dPygo histone-binding ability is dispensable for male fertility in *Drosophila melanogaster*.** (A) Amino acid sequence of dPygo PHD. The fundamental residues for H3K4me2/3 binding are in red. Note the F in position 773 (which takes the place of a W in other organisms, Fig. 1A) (Kessler et al., 2009). (B) Genotypes of male flies used in the breeding experiment: the transgenes lie on the second chromosome, whereas the endogenous *pygo* locus is on the third. On the right: the number of single males capable of having progeny, over the total number of males tested for each genotype. (C) The number of eggs per female, laid in a 24-hour time interval. MUT represents the average of the viable progeny obtained from the two different mutant transgenic flies.

2004b; de la Roche and Bienz, 2007). It is therefore plausible that in mouse testes, the ability of Pygo2 to bind to H3K4me2/3 is necessary to orchestrate or facilitate Wnt/β-catenin-dependent transcription. To test this hypothesis, we crossed MUT mice with *BATgal*<sup>+</sup> mice, bearing a well-characterized Wnt reporter transgene, that allows to detect *in vivo* Wnt signaling activity by monitoring β-galactosidase expression (Maretto et al., 2003). With a specific anti-β-galactosidase antibody we marked the cells, within the seminiferous tubules, in which the Wnt pathway is active (Fig. 4A). Neither expression levels nor number of β-galactosidase-positive cells were altered in the *MUT;BATgal*<sup>+</sup> mouse testis (Fig. 4A, compare upper with middle panels), indicating that Wnt/β-catenin-dependent transcription occurs normally. To more precisely assay this we measured by quantitative RT-PCR the expression of the reporter transgene; β-galactosidase (*lacZ*) mRNA levels were equivalent in MUT and wild-type testes (Fig. 4A, right panel). Moreover, by quantitative RT-PCR we could not detect any difference in the expression of common endogenous putative Wnt targets (Fig. 4B).

We also investigated whether other tissues exhibit subtle effects regarding the transcription of Wnt target genes. We chose three tissues: (1) the fetal liver, the major hematopoietic tissue during embryonic development, in which *Myc* and *cyclin D1* (*Ccnd1* – Mouse Genome Informatics) have been shown to be direct Lef1 targets (Müller-Tidow et al., 2004) (Fig. 4B); (2) the kidney, where *Lef1* and *Snail* are activated upon β-catenin activation (Wang et al., 2011) and where *cyclin D1* is downregulated upon loss of Pygo1/2 (Schwab et al., 2007) (data not shown); and (3) MEFs, which can be exposed to Wnt ligands when cultured (Fig. 4C). In all cases we could not detect any significant alteration in the expression of Wnt target genes (Fig. 4B,C). Taken together, these data demonstrate that the Pygo2-H3K4me2/3 interaction is not relevant for, and does not influence, the murine Wnt signaling pathway *in vivo*.



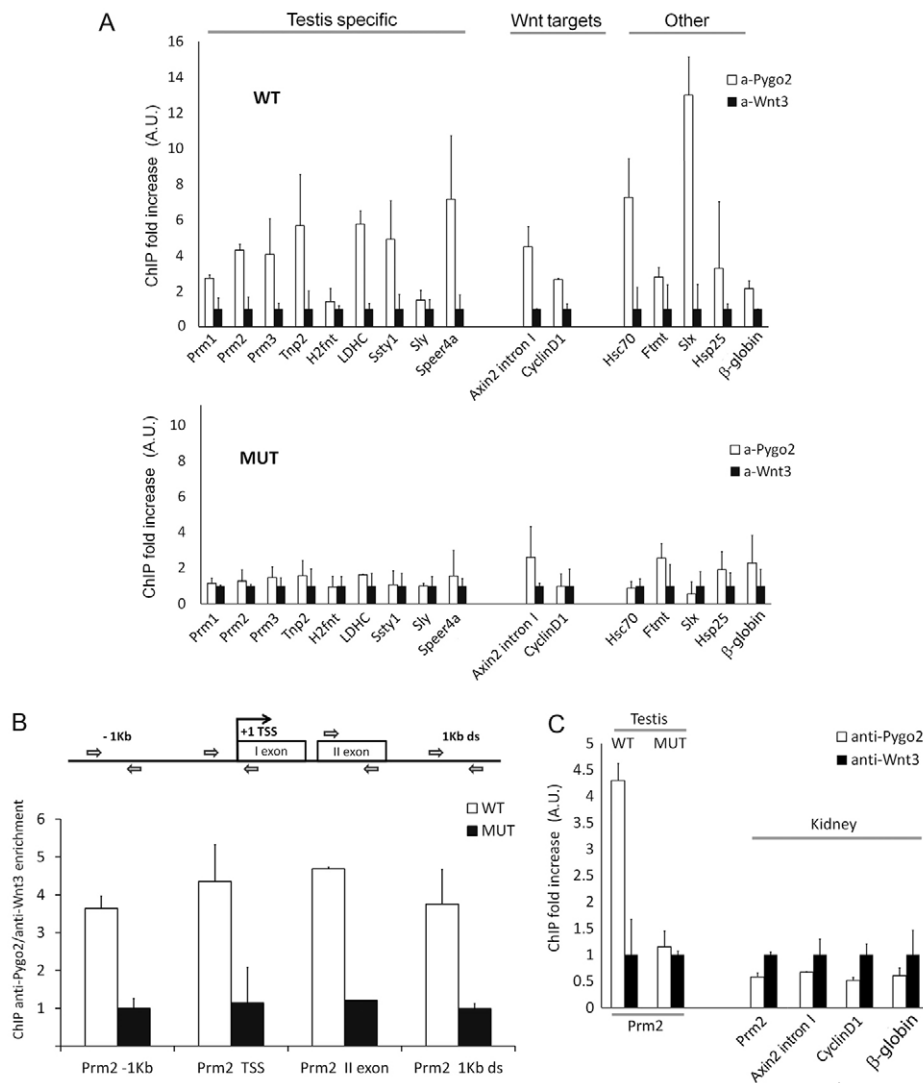
**Fig. 4. The histone-binding mutation does not influence Wnt signaling.** (A)  $\beta$ -galactosidase staining with a specific antibody (green) in a *BATgal*<sup>+</sup> mouse produces a specific bright signal (upper panels), indicating Wnt pathway transcriptional activation in mouse testes; the signal is not reduced in the sections of MUT animals (middle panels). The use of the secondary antibody alone as control allows to discriminate the background signal (lower panels). Quantitative RT-PCR confirms an equal  $\beta$ -galactosidase (*lacZ*) mRNA expression between wild type and MUT (right panel). (B) Quantitative RT-PCR shows no significant difference in the expression of known Wnt target genes between wild-type and MUT testes (left panel) and in the embryonic liver, the major hematopoietic site during development (right panel). (C) MEFs were treated for 24 hours with Wnt3a-conditioned medium, then harvested and the RNA extracted for the analysis: wild type and MUT display comparable activation of the Wnt target genes *Axin2* (not detectable previous to the stimulus) and *Lef1*. A.U., arbitrary units; n.d., not detectable; WT, wild type.

### Pygo2 binds chromatin *in vivo* in a PHD-dependent manner

We reasoned that Pygo2, when mutated in its histone-binding ability, might no longer be able to accomplish its testis-specific functions for which binding at specific genomic regions is required. To test this hypothesis we performed chromatin immunoprecipitation (ChIP) experiments on dissociated testis cells. Strikingly, in a wild-type situation we detected extensive binding of Pygo2 within the promoter of most of the genes analyzed: these were found to be enriched in the immunoprecipitated samples when using an anti-Pygo2 antibody, ranging from 2- to 12-fold increases compared with control antibodies (Fig. 5A, upper panel). A plethora of genes emerged as potential direct Pygo2 targets: among them the

testis-specific genes such as protamines, *Ldhc*, *Ssty1* and *Speer4a*, as well as others with unrelated biological function, distributed on both sex and autosomal chromosomes (Akerfelt et al., 2008) (Fig. 5A). Interestingly, putative Wnt-responsive regions in, for example, the *cyclin D1* promoter and the first intron of *Axin2* (Tetsu and McCormick, 1999; Jho et al., 2002), displayed a significant enrichment of Pygo2 binding, which was, however, not larger than the one found in other regions. This is in agreement with the apparent absence of a specific connection between Pygo2 and Wnt signaling in testes, and raises the possibility that in testis most of the Pygo2 binding across the genome is Tcf/Lef1 independent.

ChIP experiments also show that the A342E mutation causes a massive impairment in Pygo2 binding on all the regions tested. This



**Fig. 5. Pygo2 binds the chromatin *in vivo*.** (A) ChIP followed by RT-PCR. Upper panel: enrichment of several genomic regions is found upon wild-type Pygo2 immunoprecipitation, when compared with immunoprecipitation with a control Wnt3 antibody (set to 1). The genes in the charts are separated according to their biological function rather than their chromosomal positions. Lower panel: Pygo2 binding was specifically lost in the MUT situation. Note that the enrichment found on Wnt target loci is equally lost, further supporting a clear-cut separation of the histone-binding function of Pygo2 with its supposed role as Wnt signaling component. (B) Pygo2 binding is spread over the whole genomic locus spanning the *Prm2* gene: the relative position of the primer pairs used (white arrows) is indicated above the chart. (C) ChIP was performed using kidney-derived wild-type cells. In the assay, cells from wild-type and MUT testes were included as positive and negative control, respectively. The locus-specific amplification from the control Wnt3 immunoprecipitated sample is set to 1. A.U., arbitrary units; ds, downstream; WT, wild type.

observation further validates the functionality of the mutation generated, and shows that the broad binding of Pygo2 on the chromatin is dependent on an intact PHD (Fig. 5A, lower panel).

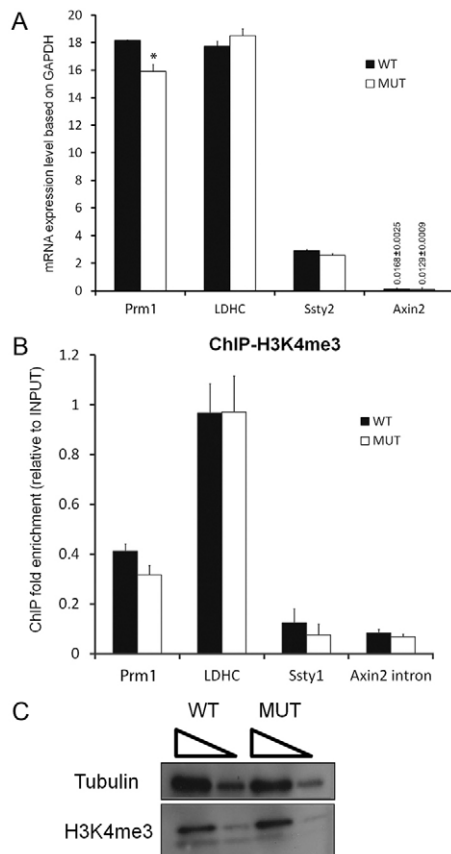
The widespread binding of Pygo2 prompted us to investigate whether its presence is specific to regulatory regions (e.g. promoters) or if it can be also found elsewhere. As represented in Fig. 5B, we designed specific primers for different positions along the *Prm2* gene locus, from 1 kb upstream of the transcriptional start site (TSS), to 1 kb downstream of the 3' UTR. The ChIP assays revealed equal binding of Pygo2 along the locus. The enrichment at every position strongly decreased in the MUT tissue (Fig. 5B). Interestingly, an equivalent enrichment of Pygo2 was found 2 and 3 kb upstream of the *Prm2* TSS, within the intergenic region equidistant between the *Prm1* and *Prm2* coding sequences (supplementary material Fig. S5). These observations suggest that Pygo2 behaves in the testis more like a chromatin-bound structural protein, rather than as a classic transcription factor.

The Pygo2-A342E mutation causes a testis-specific defect but does not lead to any obvious abnormality in other tissues. This raised the possibility that Pygo2 binds chromatin only in the testis. We sought to address this via ChIP, using dissociated cells from the kidneys, in which Pygo2 displays the highest expression (Li et al., 2004). ChIP experiments, performed parallel to testes-derived

chromatin as a positive control, showed that in kidney cells Pygo2 was not associated with the same genomic target regions as in testes. This includes the regulatory regions of Wnt target genes, such as the *cyclin D1* promoter and the first intron of *Axin2* (Fig. 5C). Although somewhat unexpected, this is consistent both with a lack of any phenotypic defect in the MUT kidney and the finding that very few differences in the expression of known Wnt target genes was measured in the kidney of *Pygo1/2* null mutants (Schwab et al., 2007).

### Loss of Pygo2 chromatin binding does not affect target gene transcription

Although ancillary modes of action are possible, dPygo seems to work as a transcriptional coactivator (Kramps et al., 2002; Carrera et al., 2008; Mieszczynek et al., 2008). However, in mouse testes, it has been proposed that Pygo2 functions as a chromatin remodeler, important for proper chromatin acetylation in terminally differentiating sperm cells (Nair et al., 2008; Jan et al., 2012). To test if the association of Pygo2 with the regulatory regions of genes affects their transcription, we selected a handful of genes on whose promoters Pygo2 shows a strong and reproducible binding, and measured whether their transcription is affected when Pygo2 binding is abrogated. Quantitative RT-PCR analyses (Fig. 6A)



**Fig. 6. Pygo2 does not control transcription in testes.** (A) Quantitative RT-PCR shows that no general effect on transcription is obtained upon abolishing Pygo2 histone-binding ability. The expression of a few selected genes, based on the GAPDH mRNA level, is represented. A statistical significance of  $P < 0.05$  is indicated by an asterisk. (B) ChIP experiments using an H3K4me3 antibody indicates that Pygo2 loss from a target locus does not influence the presence of chromatin markers associated with active transcription. The enrichment at target loci is calculated based on the input samples. (C) Western blot using decreasing protein extract dilutions of wild-type and MUT testes evidences no detectable difference in the total level of H3K4 trimethylation; tubulin antibody is used as a loading control. WT, wild type.

revealed that transcription of none of the selected genes was altered. *Prm1* was slightly downregulated; however, we interpret this as reflecting the loss of terminally differentiated cells, where *Prm1* is specifically expressed.

In addition, we performed a ChIP assay using an antibody against H3K4me3, a well-established marker for actively transcribed genes (Fortschegger and Shiekhattar, 2011). As expected, we found that H3K4me3 levels positively correlate with the transcriptional level. This was not significantly altered in the MUT testes at any of the loci tested (Fig. 6B). Hence Pygo2 binding at the tested loci does not influence the level of transcription of nearby genes; moreover, our results suggest (Fig. 6C) that Pygo2 also does not affect the H3K4 methylation level.

### The HAT enzyme Gcn5 displays a reduced association to the chromatin in the MUT testis

Pygo2 has been recently found associated with histone acetyltransferase (HAT) activity in the testis (Nair et al., 2008).

Concomitantly, in breast cancer cell lines, Pygo2 was detected to be physically associated with the Gcn5-containing STAGA HAT complex (Chen et al., 2010). By quantitative RT-PCR we measured *Kat2b/Gcn5* mRNA, and found it highly expressed in both wild-type and MUT testes (Fig. 7A). Taking these observations together, we anticipate the possibility that Pygo2 mediates the acetylation of histones in a Gcn5-dependent manner. This raises the prediction that the abrogated Pygo2-chromatin interaction leads to a decreased association of Gcn5 to the chromatin.

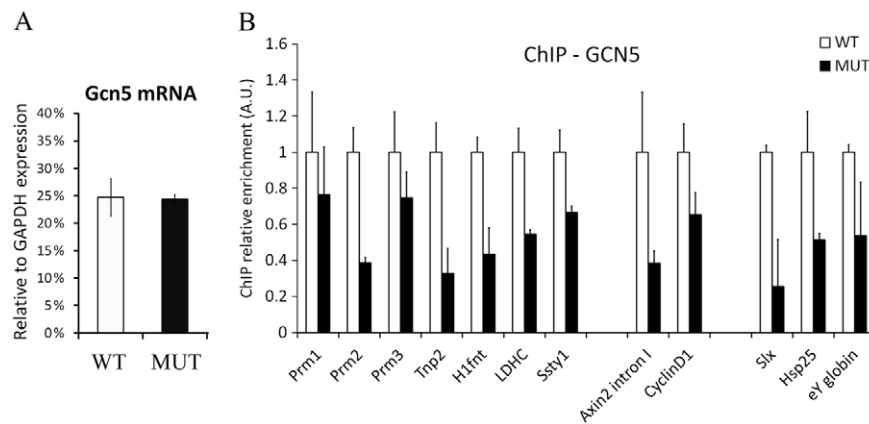
We performed ChIP using a specific anti-Gcn5 antibody, and quantified by qPCR the enrichment of the same genomic loci that we tested for Pygo2 (see Fig. 5). Of note we found that Gcn5 is associated with the chromatin in mouse testes and that its association, with a certain degree of variability, is constantly decreased in the MUT testis when compared with wild type, for every locus tested (Fig. 7B). This observation lends support to a model in which Pygo2, in testis, recruits the HAT enzyme Gcn5 to the chromatin, ensuring adequate acetylation required in late stages of sperm cell differentiation.

### DISCUSSION

The PHD is a module for binding H3K4me2/3 and is often present in proteins that coordinate the crosstalk between chromatin remodeling and transcriptional complexes (Musselman and Kutateladze, 2011). Pygo proteins have an evolutionarily conserved PHD, and they have been shown to bind, at least *in vitro*, to H3K4me2/3 (Fiedler et al., 2008; Kessler et al., 2009). However, the *in vivo* relevance of this potential association has so far not been tested outside of *Drosophila* (Fiedler et al., 2008; Kessler et al., 2009). Here we exploited the knock-in technology to generate a Pygo2-A342E mouse line, which encodes a mutated version of the protein that cannot bind to methylated histones. The mutant allele remains expressed at wild-type levels, and the resulting Pygo2-A342E protein also localizes to the nucleus like the wild-type protein (Fig. 2D). This mouse line represents an excellent system to assay for a possible *in vivo* relevance of the chromatin-binding ability of Pygo2.

Unexpectedly, mutant mice proceed normally through development, even in the absence of the potentially redundant Pygo1. This finding is reminiscent of experiments in flies, which revealed that the dPygo-H3K4me2/3 interaction is entirely dispensable in *Drosophila melanogaster* (Kessler et al., 2009). However, in contrast to *Drosophila*, the mouse Pygo2-H3K4me2/3 interaction appears to be involved in spermiogenesis. Our data and previous data suggest that in mouse testes Pygo2 does not contribute to the promotion of Wnt-signaling-related transcription; rather it acts as a structural protein and enhances the acetylation of histone tails. The chromatin hyperacetylation is believed to be crucial for the displacement of the canonical histones with the testis-specific ones, i.e. transition proteins (Tnps) and protamines (Prms), ensuring the proper condensation of the chromatin necessary for the terminal maturation of sperm cells (Govin et al., 2004). Pygo2 has been postulated to take part in this process (Jan et al., 2012), and our data show that the histone-binding ability of Pygo is needed to mediate this. Of note, a hypomorphic Pygo2 mutation (Nair et al., 2008) leads to a more severe spermiogenesis defect than the one we observed by specifically abrogating the interaction with H3K4me2/3. This probably reflects the differences in the nature of the mutations and suggests that part of the role of Pygo2 during spermiogenesis is not mediated by its direct binding to the chromatin. Clearly, however, histone binding via the PHD is important for spermiogenesis.





**Fig. 7. Decreased Gcn5 association with the chromatin in MUT testes.** (A) Quantitative RT-PCR showing the mRNA expression level of the HAT enzyme Gcn5. The values are expressed as percentage of the GAPDH mRNA, and they represent the average of two wild-type and two MUT independent testes mRNA extractions. (B) ChIP using a specific anti-Gcn5 antibody, followed by quantitative PCR. The enrichment obtained in the wild type has been set to 1 for each locus tested. A.U., arbitrary units; WT, wild type.

In mammals, PHD-containing proteins have long been implicated in chromatin-remodeling-dependent transcriptional regulation (Aasland et al., 1995). The ‘histone code reader’ hypothesis anticipates that modifications on histone tails occur sequentially, and are brought about by different chromatin-binding proteins, that guarantee the appropriate transcriptional level of a particular gene (Bienz, 2006). For example, the nucleosome-remodeling factor (NURF) is an ATP-dependent chromatin-remodeler, whose subunit bromodomain and PHD finger transcription factor (BPTF) mediates the association on the *HOXC8* promoter, and is then directly implicated in HOX gene expression during development (Wysocka et al., 2006).

The presence of a PHD finger in Pygo proteins stimulated speculations concerning its potential role in the transcriptional regulatory function of Pygo. For example, by mediating the binding to Lgs/Bcl9 as well as with the chromatin, the Pygo-PHD might, at Wnt target loci, recruit and/or stabilize the interaction of transcriptional co-factors with the C-terminal region of  $\beta$ -catenin (Valenta et al., 2011; Valenta et al., 2012; Mosimann et al., 2006). However, our data contradict the idea that the Pygo-chromatin association has any effect on transcription, and instead suggest that the binding to H3K4me2/3 is dispensable for the transcriptional regulation of Wnt target genes. Even in sperm cells, where Pygo2 binds to the regulatory regions of several genes (both Wnt targets and others) (Fig. 4), its presence or absence does not influence the transcription of genes lying close by. Although we cannot exclude subtle effects on Wnt signaling, the absence of an effect during development and tissue homeostasis (aside from the testes) suggests these differences will be negligible. Of note, our ChIP experiments revealed that the chromatin association of Pygo2 is not limited to the regulatory regions: we arbitrarily designed primers spanning the *Prm2* gene locus, and found Pygo2 was equally bound at the promoter, over the coding sequence, and in the intergenic region between *Prm1* and *Prm2* (Fig. 4B; supplementary material Fig. S5). Hence Pygo2 does not act as a common transcription factor (i.e. binding a particular consensus sequence located within a defined regulatory region). Rather, this observation indicates that Pygo2 has a structural role, possibly taking part in the massive chromatin reorganization required for germ cell terminal differentiation.

A further interesting finding is the apparent tissue specificity of the Pygo2-chromatin association. Even though the kidney is the tissue in which Pygo2 seems to be expressed at the highest level (Li et al., 2004), and despite kidney-derived protein extracts showing a strong Pygo2-chromatin association *in vitro* (Fig. 1D), in contrast to the testes, we could not detect *in vivo* binding of Pygo2 to the chromatin of kidney cells (Fig. 4C). We speculate that a testis specific co-factor

might permit/facilitate the interaction of Pygo2 with the chromatin. Alternatively, outside the testis, some Pygo2-interacting proteins might compete with H3K4me2/3 for Pygo2 binding.

Regarding the role of Pygopus proteins in Wnt signaling: in the testis Wnt signaling sustains the proliferation of spermatogonial stem/progenitor cells (Golestaneh et al., 2009), whereas Pygo2 is mainly expressed in the elongating/condensing spermatids (Nair et al., 2008). The spatial separation of Pygo2 from other Wnt signaling components might allow it to independently interact with chromatin, thereby executing another function; this function is dependent on histone binding and involves their acetylation. Interestingly, it was recently shown that, in human breast cancer cell lines, PYGO2 interacts with the histone methyltransferase (HMT) MLL2 and with STAGA, the GCN5-containing HAT complex (Chen et al., 2010). We found that association of Gcn5 with chromatin is reduced in the Pygo MUT situation (Fig. 7B), providing a potential mechanism that explains why Pygo2 is crucially involved in the histone acetylation pattern during terminal maturation of sperm cells. In fact, Gcn5 has been found to acetylate K9 and K14 on histone H3 and, albeit to a lesser extent, K8 and K16 on histone H4 (Grant et al., 1999; Kuo et al., 1996). A detailed analysis of the potential Pygo2 functional interactions with HAT enzymes in differentiating sperm cells, as well as their potential relevance for male infertility in humans, is therefore an interesting potential next step.

In conclusion, our data clearly show that the histone binding potential of the PHD of Pygo proteins is not needed in Wnt signaling in the mouse. Rather, the consequences of abrogating the interaction are restricted to a late step in spermiogenesis. In an expanding number of contexts in which Pygo2 is being implicated as a modulator of Wnt signaling (Mosimann et al., 2009; Jessen et al., 2008; Chen et al., 2010), our work provides an important piece of the puzzle needed for a holistic understanding.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

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