TDP-43 facilitates milk lipid secretion by post-transcriptional regulation of Btn1a1 and Xdh

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Milk lipid secretion is a critical process for the delivery of nutrition and energy from parent to offspring. However, the underlying molecular mechanism is less clear. Here we report that TDP-43, a RNA-binding protein, underwent positive selection in the mammalian lineage. Furthermore, TDP-43 gene (Tardbp) loss induces accumulation of large lipid droplets and severe lipid secretion deficiency in mammary epithelial cells to outside alveolar lumens, eventually resulting in lactation failure and pup starvation within three weeks postpartum. In human milk samples from lactating women, the expression levels of TDP-43 is positively correlated with higher milk output. Mechanistically, TDP-43 exerts post-transcriptional regulation of Btn1a1 and Xdh mRNA stability, which are required for the secretion of lipid droplets from epithelial cells to the lumen. Taken together, our results highlights the critical role of TDP-43 in milk lipid secretion, providing a potential strategy for the screening and intervention of clinical lactation insufficiency.
Mammalian milk not only delivers nutrients to successfully support offspring but also provides sufficient immunoregulatory agents for antimicrobial protection and neonatal survival. However, little attention has been devoted to characterize key genes involved in milk secretion in mammals. In humans, increasing evidence has demonstrated that breastfeeding is highly beneficial for infants, including reduction in the incidence of diarrhea and pneumonia, decreased risk of obesity, and increased immune system maturation. Moreover, breastfeeding can protect mothers against cardiovascular disease, metabolic syndrome, and breast cancer. Despite this, only about 20% of women maintain exclusive breastfeeding for 6 months, with lactation insufficiency being the most cited reason for this finding. Therefore, understanding the milk secretion process to improve early diagnosis and lactation performance is an urgent issue.

Among the components of milk, lipids are remarkable sources of energy for offspring in most mammals, and successful lipid secretion is critical for newborn survival during lactation. As major components of the milk lipid droplet (LD) membrane for lipid secretion, butyrophilin 1a1 (BTN, encoded by the Btn1a1 gene) is important for milk lipid secretion and therefore neonatal survival. Xanthine oxidoreductase (XOR, encoded by the Xdh gene) is also reported to modulate milk lipid secretion during lactation. However, although some studies have found that XOR can be regulated by various factors at the transcriptional level, little attention has been paid to post-transcriptional regulators. Several reports have suggested that regulation of mRNA stability or regulation at the post-transcriptional level may be the key to lactation activation. RNA-binding proteins (RBPs) mediate key steps in post-transcriptional regulation of gene expression. Therefore, identifying those RBPs that control the post-transcriptional expression of essential genes in lactation would be helpful for delineating the milk secretion process.

Here, we perform likelihood ratio tests of RBPs to screen the potential regulators of lactation and find that TDP-43 experiences positive selection in mammals. Furthermore, KO of Tardbp (TDP-43 gene) in mice results in LD secretion failure, and thereafter lactation failure and poor newborn survival. The clinical samples from lactating women further emphasize the substantial role of TDP-43 in milk secretion. For the underlying mechanism, we show that TDP-43 could bind to the 3'-untranslated regions (UTRs) of the Btn1a1 and Xdh transcripts and thereby regulate their messenger RNA (mRNA) stability. Importantly, our findings highlight the crucial role of TDP-43 in milk lipid secretion.

## Results

### Positive selection of TDP-43 gene during mammalian evolution

Lactation is a crucial physiological factor in mammalian survival. Despite some studies suggesting that post-transcriptional regulation of lactation is important, little information is currently available on the functional roles of regulators on lactation at the post-transcriptional level. As RBPs mediate key steps in the post-transcriptional regulation of gene expression, we focused on identifying those RBPs essential for the regulation of lactation. Considering that lactation is a highly characteristic feature of mammals compared with other species, we identified lactation-related genes by calculating positive selection signals in the ancestral branch of mammals, as positively selected genes in these branches may be associated with mammalian characteristics in comparison to those of other animals (e.g., fish, birds, reptiles). We therefore performed phylogenomic analysis of positive selection in 15 vertebrate genomes to identify candidate RBPs for lactation. Likelihood ratio tests (LRTs) were first employed to identify genes under positive selection in the list of mammalian RBPs. After stringent filtering, 60 one-to-one orthologous groups of RBPs across 15 species were analyzed (Supplementary Fig. 1 and Supplementary Table 1). The LRTs from the branch-site model showed that, in the ancient branch of mammals, TARDBP and SRSF9 underwent significant positive selection, with p values of 0.031 and 0.022 after false discovery rate correction, respectively. In addition, the dN/dS values were 0.27 and 12.41, respectively (Table 1). The multiple alignments and conserved codons of TARDBP and SRSF9 are shown in Supplementary Figs. 2 and 3, respectively, suggesting that TARDBP and SRSF9 may play important roles in mammalian features compared with other animals.

TDP-43 loss in mammary epithelium induces lactation failure. As milk secretion is one of the most characteristic features of mammalian species, and TDP-43 (encoded by the TARDBP gene) is involved in breast cancer progression, we hypothesized that TDP-43 may play an important role in milk secretion, while the other candidate, SRSF9, was excluded for further investigation because of its negatively regulatory roles on milk secretion-related genes (BTN and XOR) (Supplementary Fig. 4A, B). The expression pattern of TDP-43 at different mammalian mammary gland stages was first examined in mice. Immunohistochemical assay showed that the TDP-43 protein was highly expressed during late pregnancy and early lactation in comparison with that during virginity and involution (Fig. 1a, ×40 objective magnification; and Supplementary Fig. 4C, ×10 objective magnification). These results were confirmed by quantitative real-time polymerase chain reaction (qRT-PCR, Supplementary Fig. 4D) and western blot assays (Supplementary Fig. 4E, F). TDP-43 was located in both the myoepithelial and luminal cell layers, as indicated by its colocalization with cytokeratin 14 and 18 (CK14 and CK18) (Supplementary Fig. 4G, respectively).

To examine the roles of TDP-43 in the mammary epithelium during pregnancy and lactation, we disrupted TDP-43 expression in Tardbp floxed mice using transgenic mice expressing Cre recombinase (Cre) driven by the whey acidic protein (WAP) promoter activated during middle pregnancy to lactation in luminal epithelial cells. The WAP-Cre line abrogated TDP-43 expression effectively at pregnancy day 17.5 (P17.5) and at lactation day 10 (L10), confirming Tardbp knockout (KO) in the mammary epithelium (Supplementary Fig. 5A–C). To characterize the overall survival of offspring, we calculated the survival rate of pups from various litter sizes. First-litter pups born to Tardbp KO female mice (Tardbpflploxflplox/WAP-Cre, shown as Tardbp−/− mice) showed obviously lower survival rates in comparison to those born to Tardbp-intact mice (WAP-Cre, shown as wild type (WT) in the figures) (Supplementary Fig. 5D). Although the pup survival rate improved in the KO group during the second lactation, the overall trend was consistent with that of the first gestation (Supplementary Fig. 5E). To exclude discrepancy in nourishment caused by different sized litters, the pup survival rate was analyzed for the same sized litters (6, 7, or 8 pups per litter) (Fig. 1b, c and Supplementary Fig. 5F), which showed a similar pattern of declining pup survival.

At the end of L2, we adjusted the litter size to seven to observe the weight of the surviving pups. Although most pups died before L2, those pups that survived lived until L13, after which time the survival rate markedly decreased from L15 to L23 (Fig. 1d). The survival rate using unadjusted litter sizes also exhibited a similar decline after L15 (Supplementary Fig. 5D). Furthermore, the average weights of the surviving pups born to Tardbp−/− mothers were substantially lower than those born to WT mothers during the first lactation (Fig. 1e, black lines) and second lactation (Fig. 1f). To
exclude possible defects in pups, pups born to WT and Tardbp−/− mothers were cross-fostered by Tardbp−/− and WT mothers, respectively. Results demonstrated that pups born to Tardbp−/− female mice but fostered by WT mice beginning at L2 grew with normal body weights and survival rates, whereas pups born to WT females but fostered by Tardbp−/− mice exhibited reduced growth (Fig. 1e, blue lines and Supplementary Fig. 5f). A similar reduction in body weight was observed when pups born to WT females were fostered by Tardbp−/− females at the beginning of L10 (Fig. 1e, red lines). These results indicate that TDP-43 is required for lactation to support offspring survival in mice. To ensure that the observed failure of offspring growth and viability was dependent on the mothers, rather than that of the pups themselves, we genotyped all deceased pups and found normal genotype distribution (Supplementary Fig. 5f). Overall, these results demonstrate that maternal Tardbp−/− induced failure of both pup viability and growth.

**TDP-43 loss results in disrupted lipid secretion.** Morphological changes were first evaluated to determine the possible causes of lactation defects in Tardbp KO mice. Mammary glands from Tardbp−/− mice harvested at the mid-pregnancy (P15.5, P17.5) to lactation stages (L2 and L10) demonstrated no differences in either lobuloalveolar structures or alveolar densities compared with WT mice based on whole-mount (Supplementary Fig. 6A) and hematoxylin and eosin (H&E) staining (Supplementary Fig. 6B). Moreover, TUNEL assay and Ki67 staining revealed indistinguishable rates of apoptosis and proliferation at P17.5 (Supplementary Fig. 6C–F). These results suggest that pup deaths were unlikely due to structural defects caused by Tardbp KO.

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**Table 1 Positive selection on mammalian TARDBP and SRSF9.**

| Gene   | lnL0   | lnL1   | ΔlnL   | dN/dS   | Positive selection site |
|--------|--------|--------|--------|---------|-------------------------|
| TARDBP | −5443.244 | −5437.227 | 12.04*  | 371.37  | 352P*, 360Q             |
| SRSF9  | −1846.979 | −1852.675 | 11.39*  | 12.41   | 1N, 22V, 44R, 129R, 159R |

*lnL: log likelihood, lnL0: lnL under model A null hypothesis, lnL1: lnL under model A alternative hypothesis

*Five percent significance level
To identify factors contributing to the death of pups nursed by Tardbp KO mice, we focused on milk, the main energy source for offspring survival. Milk was collected from lactating mice for volume and compositional analyses. Tardbp KO led to a significant decrease in milk secretion compared to that in WT mice at L2 and L10 using two concentrations of oxytocin (the higher dose to remove all milk at an early stage and the lower dose at an established lactation in conventional use), respectively (Fig. 2a and Supplementary Fig. 7A). We next measured changes in milk protein and lipid compositions, which are the predominant sources of nutrition in milk. The protein quantitation assays demonstrated that the protein compositions of milk collected from lactating Tardbp−/− and WT mice at L2 were indistinguishable (Supplementary Fig. 7B, C), and the expression levels of several essential genes related to milk protein in mammary gland epithelial cells (MECs) were unaffected by Tardbp loss (Supplementary Fig. 8A–C). However, the concentrations of triacylglycerols (TAGs), the predominant component of lipids, were markedly lower in milk taken from Tardbp−/− mice compared with that from WT mice (Fig. 2b). Polyunsaturated fatty acids and essential fatty acids, which are indicators of exogenous uptake, were similar between the milk from Tardbp−/− and WT mice (Supplementary Fig. 7D, E), suggesting that uptake by the mice was the same. These observations suggest that loss of TDP-43 can lead to the abnormal composition of milk lipids but not of milk proteins.

To further clarify lipid disorder induced by Tardbp KO, we collected MECs to analyze their lipid concentrations. Interestingly, although TAG concentrations in milk were markedly lower in Tardbp−/− mice (Fig. 2b), the cellular TAG concentrations in MECs were much higher in Tardbp−/− mice than that in WT mice at L2 and L10, respectively (Fig. 2c, d). These results indicate that the phenotype observed in our study was likely due to unsuccessful milk lipid secretion from MECs to the outside, resulting in the accumulation of higher TAG concentration in MECs but lower TAG concentration in milk. Thus, these results indicate that lipid secretion was affected by Tardbp KO.

**TDP-43 loss results in large LD accumulation.** Because milk lipids are secreted as milk fat globules (MFGs) wrapped in a bilayer plasma membrane, we compared MFGs between WT and Tardbp−/− mice. Results indicated that MFGs from Tardbp−/− mice were substantially larger than those from WT mice at L2 and L10 (Fig. 3a, b). Moreover, milk alveolar structures and MECs from Tardbp KO mice were clearly different from those in WT mice at L2 and L10 (Fig. 3a, b). Polyunsaturated fatty acids and essential fatty acids, which are indicators of exogenous uptake, were similar between the milk from Tardbp−/− and WT mice (Supplementary Fig. 7D, E), suggesting that uptake by the mice was the same. These observations suggest that loss of TDP-43 can lead to the abnormal composition of milk lipids but not of milk proteins.

**Fig. 2 Milk secretion is impaired in Tardbp−/− female mice. a** Analysis of milk volume from mammary glands of wild-type (WT) and Tardbp−/− female mice following 10 U of oxytocin stimulation (n = 7 for each genotype). b Analysis of triacylglycerols (TAGs) in milk collected from WT and Tardbp−/− mice at lactation day 2 (L2) (n = 6 for each genotype). c, d Analysis of TAGs in mammary gland epithelial cells (MECs) collected from WT and Tardbp−/− mice at L2 (C) (n = 5 for each genotype) or L10 (D) (n = 6 for each genotype). Data are means ± SD (a) or means (b–d). TAG levels in MECs were normalized to protein concentration of each sample. Unpaired t test was used to evaluate statistical significance. *P < 0.05, **P < 0.001. Source data are provided as a Source Data file.
Fig. 3 Larger lipid droplets are present in MECs after knockout of Tardbp. a, b Phase contrast micrographs (a) and statistics (b) of milk fat globules (MFGs) from wild-type (WT) and Tardbp−/− mice analyzed using Image-Pro Plus 5.0 at lactation day 2 (L2) (left, n = 7 mice) or L10 (right, n = 6 mice). Scale bar: 100 μm. Data are means ± SD. Unpaired t test was used to evaluate statistical significance. ***P < 0.001. c Hematoxylin and eosin staining of mammary glands from WT and Tardbp−/− mice at L1, L2, and L10. Magnified areas at L10 are shown in red boxes (left). Arrowhead shows cytoplasmic lipid droplets (CLDs) in cells and arrow shows large lipid droplets (LDs) in alveolar lumen. Scale bar: 20 μm. d Immunofluorescence staining of adipophilin (PLIN2) (red) showing LDs in alveoli of mammary glands from WT and Tardbp−/− mice at L1 and L2. Sections were also stained with wheat germ agglutinin (WGA) (green) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to outline luminal border or nuclei. Magnified areas (right) are shown in white boxes (left). Scale bar: 10 μm. e Electron microscopy images of mammary gland sections from WT and Tardbp−/− mice at L1. Arrowhead indicates nucleus and asterisk shows lumen of mammary glands. Scale bar: 2 μm. Source data are provided as a Source Data file.
lactation32. The LDs observed in the alveoli were indeed larger in size and accumulated around the edges of the lumen during lactation in Tardbp−/− mice (Fig. 3d and Supplementary Fig. 9B). Moreover, electron microscopy images of mammary gland sections from WT and Tardbp−/− mice further demonstrated that large LDs were accumulated in the MECs (Fig. 3e). Taken together, these data indicate that loss of TDP-43 resulted in the accumulation of large LDs in the MECs, which may eventually lead to lactation failure.

TDP-43 loss reduces BTN and XOR expression. To explore the mechanism of lipid secretion disorder in Tardbp−/− mice, RNA-sequencing (RNA-seq) was performed to identify the molecular processes at L1 between WT and Tardbp−/− mice. Among the differentially expressed genes identified in the Tardbp KO epithelium (Fig. 4a), 44 genes were involved in lipid metabolism. It has been reported that the TDP-43 protein regulates downstream genes by directly binding to the motif of UG-enriched sequences33. Therefore, to identify genes directly regulated by TDP-43, we performed statistical analysis to determine enrichment of the UG-repeated motifs in the mRNAs of the above 44 genes (Supplementary Table 2). Results showed that Btn1a1 mRNA exhibited the most significant enrichment in the UG-repeated motif. Previous research has also shown that Btn1a1 KO mice exhibit poor pup survival and large lipid droplets13, thus phenocopying Tardbp KO mice. This suggests that Btn1a1 may be a downstream gene of TDP-43 that regulates milk lipid secretion. In addition, qRT-PCR assay confirmed the significant decrease in Btn1a1 expression in the TDP-43 KO mammary gland at L1 and L10 (Fig. 4b). Moreover, Xdh, an important interactor with BTN for milk lipid secretion34, was significantly decreased at L1 and L10 (Fig. 4c), whereas the expression of Cidea was comparable at P17.5 and L10 at both the mRNA and protein levels (Supplementary Fig. 10A–C). We therefore focused on the Btn1a1 and Xdh genes, which are well-recognized mediators of milk lipid secretion32.

To confirm the regulatory effects on Btn1a1 and Xdh, we compared their expression levels between WT and Tardbp−/− mice. The mRNA levels of Btn1a1 and Xdh decreased in the Tardbp−/− mammary gland during lactation; moreover, BTN (Btn1a1 protein) and XOR (Xdh protein) expression were both remarkably reduced at P17.5 and L1 (Fig. 4d, e). To prove the regulatory effects in vitro, knockdown of Tardbp by short hairpin RNA (shRNA) in a differentiated mammary epithelial cell line (HC11) also led to substantially lower expression of BTN and XOR at the protein level (Fig. 4f), thus suggesting a regulatory effect of TDP-43 on BTN and XOR. Knockdown of TDP-43 in the HC11 cells impaired dome formation (Supplementary Fig. 10D), which results from fluid secretion by mammary epithelial cells upon treatment with lactogenic hormones
in vitro[^35-37]. Moreover, the decrease in dome formation was partially rescued when BTN and XOR were co-expressed in cells expressing sh-TDP-43 (Supplementary Fig. 10E, F).

**TDP-43 binds to Btn1a1 and Xdh mRNA.** TDP-43 is a RBP[^38], with the binding motif of the UG-enriched sequence[^33,39] regulating RNA in a variety of ways. To decipher the regulatory mechanism of TDP-43 on *Btn1a1* and *Xdh*, we examined whether a UG- or TG-enriched sequences existed in the *Btn1a1* and *Xdh* gene promoter regions, pre-mRNA, and mRNA. Both *Btn1a1* and *Xdh* mRNA contained a large TG-enriched sequence in the 3'-UTR (Fig. 5a), suggesting that TDP-43 may bind to the 3'-UTRs of the *Btn1a1* and *Xdh* transcripts directly. To demonstrate this,

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[^35-37]: These references should be properly formatted as inline citations in the text.

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we studied the interaction between the TDP-43 protein and mRNA of Btn1a1 and Xdh by RNA immunoprecipitation (RIP). Results demonstrated that TDP-43 was bound to Btn1a1 and Xdh mRNA strongly in the MECs (Fig. 5b) and HC11 cell line (Fig. 5c). An RNA pull-down assay was also used to confirm the interaction between mRNA and protein. Both Btn1a1 (nt 2781–3398) and Xdh (nt 4399–4623) fragments containing TG-enriched sequences effectively pulled down the TDP-43 protein in the primary MECs and HC11 cells (Fig. 5d, e).

To determine which domains of TDP-43 were responsible for its interaction with Btn1a1 and Xdh mRNA, Flag-TDP-43 or deletion fragments was overexpressed for the RIP assays using differentiated HC11 cell lysates (Fig. 5f, g). The C-terminal domain of TDP-43 (C-term) could not bind to either Btn1a1 or Xdh mRNA, whereas the other deletion constructs showed clear binding affinity to both mRNAs. In addition, both the RRM1 and RRM2 domains were required for the interaction of TDP-43 with Btn1a1 and Xdh mRNA. The RRM1 domain was the predominant functional RNA-binding domain, with RRM1 deficiency (ARRM1) showing the greatest reduction in binding affinity compared with the TDP-43 full-length construct (FL) (Fig. 5j).

We further performed reciprocal RNA pull-down assays using four biotinylated Btn1a1 mRNA fragments or six biotinylated Xdh mRNA fragments (Fig. 5i) to confirm the interaction between TDP-43 and Btn1a1 and Xdh mRNA. Consistent with Fig. 5d, e, the 2781–3398 nucleotides of Btn1a1 and 4399–4623 nucleotides of Xdh containing UG repeated at the 3′ end of the mRNA strongly interacted with TDP-43, although the 1–900 nucleotides of Btn1a1 and 899–1859 and 2744–3718 nucleotides of Xdh also showed weak binding to TDP-43 (Fig. 5j).

Taken together, the above data demonstrated strong interactions between the TDP-43 protein and Btn1a1 and Xdh mRNA.

**TDP-43 loss decreasesBtn1a1 and Xdh mRNA stability.** TDP-43 can regulate mRNA stability through the 3′-UTR of mRNA. We therefore examined the decay of Btn1a1 and Xdh mRNA following treatment with actinomycin D, a RNA Pol II inhibitor, to stop transcription in primary MECs from P17.5 and L1. Results showed that Btn1a1 and Xdh mRNA declined significantly in the absence of TDP-43 between the WT group and Tardbp KO group at P17.5 and L1, and internal control Gapdh mRNA remained unchanged (Fig. 6a, b), suggesting that TDP-43 loss decreased the stability of Btn1a1 and Xdh mRNA. Moreover, overexpressed TDP-43 full-length (Flag-FL) but not the C-terminal domain lacked the RRM1 and RRM2 regions, significantly suppressing the decay of Btn1a1 and Xdh mRNA in the differentiated HC11 cell line (Fig. 6c). Both the BTN and XOR protein levels were remarkably increased upon TDP-43 overexpression compared with that in the control group (Supplementary Fig. 11A). These data indicate that TDP-43 could stabilize Btn1a1 and Xdh mRNA levels.

To further demonstrate that TDP-43 regulates Btn1a1 and Xdh mRNA stability through the 3′-UTR, we inserted the 3′-UTRs of Btn1a1 or Xdh following the green fluorescent protein (GFP) gene into a mammalian expression vector, representing GFP-Btn1a1-UTR and GFP-Xdh-UTR, respectively (Fig. 6d, upper), and then determined GFP change upon TDP-43 knockdown in the HC11 cells. As expected, knockdown of TDP-43 by two independent shRNAs led to substantially lower GFP expression in the GFP-Btn1a1-UTR and GFP-Xdh-UTR groups compared with their scramble controls (Fig. 6d), whereas GFP expression remained unchanged upon TDP-43 knockdown in the GFP-Control-UTR group. To confirm the regulation of TDP-43 on Btn1a1 and Xdh mRNA stability, we next generated deletion mutations of theBtn1a1 and Xdh 3′-UTRs without TDP-43-binding sites (GFP-Btn1a1-mutUTR and GFP-Xdh-mutUTR, respectively). RIP-qPCR assays were then conducted to confirm that deletion mutations of the UG-enriched sequences could severely abate the interaction between GFP mRNA and the TDP-43 protein (Supplementary Fig. 11B). Mutation of the TDP-43 binding site completely abolished the down-regulation of GFP expression upon TDP-43 knockdown (Fig. 6d). To measure whether TDP-43 could regulate mRNA stability of the above GFP reporters, we detected the GFP mRNA expression of each group upon actinomycin D treatment after TDP-43 knockdown. Results showed that the GFP mRNA stability of GFP-Btn1a1-UTR and GFP-Xdh-UTR, but not GFP-Btn1a1-mutUTR or GFP-Xdh-mutUTR, significantly decreased upon sh-TDP-43 treatment compared with the control group (Supplementary Fig. 11C). To determine whether TDP-43 could regulate BTN and XOR expression in humans, we introduced human BTN1A1 and XDH 3′-UTRs into GFP reporters to generate GFP-hBTN1A1-UTR and GFP-hXDH-UTR, respectively. Immunoblot analysis showed that knockdown of TDP-43 by independent shRNAs reduced GFP expression of GFP-hBTN1A1-UTR and GFP-hXDH-UTR relative to the sh-control (Supplementary Fig. 11D, E).

**TDP-43 loss results in early involution.** We also examined whether premature involution occurred during lactation in Tardbp−/− mice. The secretory lobuloalveolar structures were remodeled into glandular structures in Tardbp−/− mice, whereas the compact alveolar structures persisted in WT mice until L21. Loss of alveolar structures was also observed at L18 (Fig. 7a). Although Tardbp−/− mammary glands were similar to WT mammary glands in whole-mount staining at L15 (Fig. 7a), H&E staining at higher magnification showed that the Tardbp−/− mammary epithelium accumulated MFGs in the alveoli and exhibited cell shedding at L15 and L18 (Fig. 7b, c and Supplementary Fig. 12A). Moreover, severe tissue remodeling in the Tardbp−/− mammary epithelium eventually resulted in the collapse of many mammary alveoli at L21 (Fig. 7d). This tissue damage in Tardbp−/− mice was correlated with the time course of pup death (Fig. 1d). Moreover, we detected cell division using Ki67 staining during late lactation and found that cell proliferation did not change at L15 and L18, but decreased at L21 in KO mice (Supplementary Fig. 12B, C). The underlying mechanism requires further investigation.

Overall, these data demonstrated that depletion of Tardbp in the female mammary gland led to premature involution.

**Low TDP-43 expression relates to human lactation deficiency.** To explore the potential effect of TDP-43 expression on human milk secretion, we collected fresh milk samples from a total of 60 healthy lactating women who gave birth to a full-term infant. It has been reported that intracellular components of MECs, including mRNAs, can be trapped within MFGs during cellular MFG formation and secretion43,44, and thus mRNA expression profiles from MFGs can be representative of MEC gene expression45,46. Therefore, to analyze the expression levels of TARDBP in human MECs during lactation, MFGs from human breast milk were obtained by centrifugation for RNA isolation. We then detected the MFG mRNA levels of cell-specific markers to rule out possible contamination of RNA from immune cells (Fig. 8a), which are abundant in human milk47. Questionnaire follow-up by telephone interview was performed to assess milk secretion in the mothers, which confirmed that partial breastfeeding and formula feeding were driven by necessity. Our results demonstrated that TARDBP was significantly up-regulated in the exclusive breastfeeding group in comparison to the partial
breastfeeding group (Fig. 8b). The expression levels of HNRNPA1, another RBP, showed no changes among the different breastfeeding groups (Fig. 8c). Furthermore, we found no significant differences among groups in terms of milk collection time and delivery type (Supplementary Fig. 12D, E). This result suggests that low expression of TDP-43 may be correlated with lactation deficiency in human milk secretion.

**Discussion**

Milk secretion is critical for nutrition delivery from parent to offspring. Recently, researchers developed intravital imaging procedures using transgenic mice to provide mechanistic insight into the secretion of lipid droplets within the mammary epithelium in real time, which could be applied to investigate trafficking events during lactation. However, the
fundamental regulation of milk secretion remains largely
unknown. In the present study, we found an important parti-
cipant (TDP-43) required for milk fat droplet secretion in the
lactating mammary gland, which contributed to lipid secretion
by regulating Btn1a1 and Xdh mRNA stability. In addition,
Tardbp KO resulted in a reduction in Btn1a1 and Xdh mRNA
stability and induction of lipid secretion failure in the mam-
mary epithelium (Fig. 8d). These results highlight the the
critical role of TDP-43 in milk lipid secretion, thus enhancing our understanding of milk secretion.

Positive selection is a considerable evolutionary force behind the divergence of species50,51, and genome-wide scans for positive selection of protein-coding genes are valuable tools for gaining insight into the genomic mechanisms of traits52. In the present study, we performed phylogenomic analysis of positive selection of protein-coding genes in 15 vertebrate genomes to identify candidate genes for milk lipid secretion. Our results revealed that TARDBP in 15 vertebrate genomes to identify candidate genes for milk lipid secretion. Our results revealed that TDP-43 is involved in the progression of breast and other cancers29,57,58. In our study, TDP-43 loss resulted in large lipid droplets, consistent with the phenotypes observed in 

**Fig. 8 Low TDP-43 expression relates to human lactation deficiency.**

- **a** Quantification of mRNA expression levels of cell-specific markers in MSCs (milk somatic cells, n = 3) and MFGs (milk fat globules, n = 3). MSCs were obtained from fresh human breast milk by centrifugation. Specific markers for polymorphonuclear neutrophils (CD18 and GPR97), lymphocytes (CD3e), macrophages (CD68), and MEC genes encoding milk proteins (CSN1S1, CSN2, CSN3, and LALBA) are shown.
- **b** qRT-PCR analysis of TARDBP (b) and HNRNPA1 (c) mRNA expression levels in MFGs of fresh human milk on days 3-5 postpartum. Exclusive breastfeeding, n = 45; partial breastfeeding, n = 13; formula feeding, n = 2. d Graphic abstract of roles of TDP-43 in regulating milk lipid secretion. Data are means ± SD. Unpaired t test was used to evaluate statistical significance. *P < 0.05; n.s., not significant. Source data are provided as a Source Data file.

In the present study, many pups from Tardbp KO mothers died within the first 2 days of lactation (Fig. 1b, c), similar to the findings in Btn1a1 KO mice13. This may be due to large lipid droplets accumulating in the mammary gland, resulting in glandular ducts becoming full of fat and pups being unable to obtain milk in the first 2 days. Once milk began to flow, the pup survival rate stabilized until L15. Thereafter, there was a noticeable drop in survival, especially when we adjusted the number of pups in each litter to seven at L2. These results suggest that accumulation of lipids within the mammary epithelium impaired milk secretion in Tardbp KO mice, and thus mothers could only maintain a certain number of pups (litter size) during late lactation.

Previous research has demonstrated that global heterozygous KO of Xdh results in premature involution during lactation17. However, in a mammary-specific XOR KO model, homozygous deletion of Xdh in the mammary epithelial cells results in only a modest lactation defect19. This discrepancy may result from the different KO models: the former study applied heterozygous deletion of XOR in the whole body, whereas the latter knocked out XOR specifically in mammary epithelial cells. These studies suggest that deletion of XOR in the mammary epithelial cells alone may not result in premature involution. In the present
study, both the pre-involution phenotype and down-regulation of XOR expression were observed in conditional TDP-43 KO mice; however, we cannot conclude that pre-involution of the Tardbp KO mammary gland was only mediated by regulation of Xdh expression. It is, therefore, possible that another mechanism also contributed to this phenomenon.

Of note, TDP-43 is also involved in systemic lipid homeostasis. In liver tissue, TDP-43 interacts with LncLSTR to contribute to this phenotype. In the female mammary gland, Mechanistically, we demonstrated that low TDP-43 expression may be associated with the Ensembl (http://asia.ensembl.org/index.html) species tree was used as a chalumnae) used as background branches. The topology of the 15 species from two reptiles (Chicken (Gallus gallus), Meleagris gallopavo (Turkey), Ornithorhynchus anatinus (platypus), covering most mammalian taxonomic groups and used as foreground branches, and four birds (Anolis carolinensis, Ficedula albicollis (yellow-browed warbler), Gurney’s Plover (Pluvialis apricaria) and one fish (Lattimia chalumnae), as used background branches. The topology of the 15 species from the Ensembl (http://asia.ensembl.org/index.html) species tree was used as an input tree in the PAML test. A list of 80 human RBPs were used for evolutionary analysis to estimate the synonymous and nonsynonymous rates (Ks/da) and inference of positive selection by LRTs in the most recent common ancestor of mammals. After selecting one-to-one orthologous genes of human RBPs among the 15 species and the longest transcript of each in Ensembl, we removed the OGs, which contained less than nine orthologous genes, and which had no orthologous gene to the outgroup (Lattimia chalumnae). We used Prank55 for multiple alignment of coding sequences (CDSs) and Gblock56 to identify conserved codons with parameters of t = c. The OGs with conserved alignment sequences <200 nt were removed. The dN/dS ratios (ω) of the mammalian lineage for each gene were estimated by the branch-site model in PAML. The log likelihoods of the positive detection model (lnL1: model A alternative hypothesis) and the corresponding null model (lnL0: model A null hypothesis) were calculated on the mammalian lineage of the 60 RBPs with the following control file in PAML. Null hypothesis (branch-site model A, with ω2 = 1 fixed) (model = 2, NSsites = 2, fix_omega = 1, omega = 1); alternative hypothesis (branch-site model A, with ω2 estimated) (model = 2, NSsites = 2, fix_omega = 0, omega = 1). The false discovery rate (FDR) was calculated for multi-correction for all orthologous genes of the RBPs. Genes with FDR <0.05 were identified as positively selected genes.

Mice. The Tardbp floxed mice were generated by the gene targeting approach using the RAC targeting vector to delete of exons 2 and 3 in the Tardbp mRNA as previously reported50 and were then bred with WAP-Cre transgenic mice obtained from the Jackson Laboratory (008735). All experimental procedures and animal care and handling were performed per the protocols approved by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences.

Primary MEC preparation. Mammary glands were minced and then digested in DMEM/F12 containing 5% fetal bovine serum (FBS), 1% penicillin–streptomycin–glutamine, 300 μM collagenase I (Sigma, C0130), and 100 μM hyaluronidase (Sigma, H3506) for 1–2 h at 37 °C. After digestion, a single-cell suspension was obtained by sequential incubation with 0.25% trypsin–EDTA for 5 and 5 mg/ml insulin (Sigma, D4960) containing 0.1 mg/ml DNase I (Roche, 11249392001) for 5 min at 37 °C with gentle pipetting. Finally, red blood cells were removed in 0.8% NH4Cl, followed by filtration through a 40-mm filter. Primary MECs were grown in DMEM/F12 medium with 10% FBS.

Cell culture and mRNA stability analysis. HC11 cell line was from Bernd Groner lab, Ludwig Institute for Cancer Research. The HC11 cells were grown in RPMI-1640 medium with 10% Foetal bovine serum (FBS), penicillin–streptomycin–glutamine (Sigma, I5500), and 10 ng/ml epidermal growth factor (EGF, Gibco, P61351) for differentiation induction, confluence cells were grown for 24 h in a medium without EGF supplementation, followed by growth in DIP medium (1 μM dexamethasone, for differentiation induction, confluence cells were grown for 24 h in a medium without EGF supplementation, followed by growth in DIP medium (1 μM dexamethasone) for 4 days, and the experiments were performed in the most recent pre-infusion. Interchangeable primer set was used for qRT-PCR on a QuantStudio 3 instrument using a SYBR Green PCR Master Mix (Applied Biosystems). Primers used are listed in Supplementary Table 3.

Whole-mount staining. The fourth mammary glands were excised and spread on microscope slides and fixed in 25% glacial acetic acid and 75% ethanol for 1 h. Tissues were then stained in carmine aluminum solution overnight at 4 °C. After staining, the slides were dehydrated through increasing ethanol concentrations, cleared in xylene, and coverslipped with Neutral Balsam (Solarbio, G8590).

Histology and immunostaining. Mammary glands were fixed in formalin and embedded in paraffin. Tissue blocks were then sectioned at 5-μm thickness and stained with H&E. For immunostaining, sections were dehydrated with graded alcohol and boiled in 10 mM sodium citrate for antigen retrieval for 20 min. Sections were then used for immunohistochemistry and immunofluorescence analyses. For immunohistochemistry, sections were incubated three times with 3% H2O2 for 5 min to inactivate endogenous peroxidases, and then blocked with 10% goat serum for 2 h and incubated with primary antibodies at 4 °C overnight. The slides were then washed three times in phosphate-buffered saline (PBS) and incubated with secondary antibodies for 1 h at room temperature and developed with 3,3′-diaminobenzidine. For immunofluorescence, sections were blocked for 2 h, and then incubated with primary antibodies for 2 h and secondary antibodies for 1 h at room temperature after direct antigen retrieval. The slides were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (Vectorshad, H-1200, Vector Laboratories). The primary antibodies used in immunostaining were TDP-43 (1:500) (Abcam, ab105355), K14 (1:1000) (Abcam, ab7800), K18 (1:1000) (Abcam, ab6688), PLD2 (1:1000) (Progen, GP40), milk antibody (1:500) (Nordic Immunology, 5941), K67 (1:1000) (Abcam, ab15580), and WGA (1:1000) (Life Technologies, 11261). The secondary antibodies used in immunostaining were fluorescein-labeled antibody (1:1000) (KPL, 0.2-15-06), fluorescein-labeled anti-mouse (1:1000) (KPL, 0.2-18-06), and goat-anti-mouse (1:2000) (Life Technologies, A10521), goat anti-rabbit (1:2000) (Life Technologies, A10520), and TRITC (tetramethylrhodamine isothiocyanate) rabbit anti-guinea pig (1:2000) (Life Technologies, A18888).

Plasmid construction, knockdown, and overexpression. For knockdown, small interfering RNAs (siRNAs) were purchased from Ribobio and shRNAs were cloned into the pLKO.1 vector. The siRNA or shRNA sequences are listed in Supplementary Table 3. For overexpression, FL and mutant mouse TDP-43 with Flag tags were cloned into pTRIPZ-inducible lentiviral expression vector, which can induce overexpression under 2 μg/ml doxycycline (Sigma, D9891). Sequencing verified all plasmid constructs to exclude mutations. These vectors were co-transfected with pSAX2 and pMD2G (4:3:1) into 293 T cells to produce lentiviral particles, which were further analyzed or experiments. We used sh-GFP (Addgene #30323) and sh-TRC (Addgene #10879) as the shRNA controls and pTRIPZ-Flag-empty vector as the
overexpression control. For RNA pull-down assays, fragments of Btn1a1 and Xdh were cloned into pcDNA3.1 under the T7 promoter. To generate the GFP reporter vector (pcDNA/GFP), the GFP fragment was cloned into pcDNA3.1(−) at the BamHI and EcoRI sites. The pcDNA/GFP-Btn1a1-UTR construct was then created by inserting the Btn1a1 3′-UTR sequence (corresponding to NM_013483.3 from 2781 to 3398 nt) into the pcDNA/GFP vector at the EcoRV and SacI sites. All primers used in this study are listed in Supplementary Table 3.

**Western blot analysis.** Protein lysates were electrophoresed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were incubated in 5% nonfat dry milk for 1 h and primary antibodies at 4 °C overnight, and then incubated with horseradish peroxidase (HRP)-linked secondary antibodies (Sigma) for 1 h at room temperature. Protein expression was detected using a chemiluminescent HRP substrate (Millipore). The antibodies used for immunoblotting were: Flag (1:2000) (CST, #14793), α-tubulin (1:5000) (Sigma, T5168), GAPDH (1:2000) (Santa Cruz, sc-25778), TDP-43 (1:2000) (Abcam, ab109355), XDH (1:2000) (Abcam, ab109235), and BTM (1:1000) (Acris, AP09532SU-N). Source data are provided as a Source Data file.

**RNA pull-down.** The mRNA fragments of Btn1a1 and Xdh were synthesized in vitro using a HiScript T7 High Yield RNA Synthesis Kit (NEB, E20405). These RNAs were attached to a single biotinylated nucleotide at the 3′ terminus of the RNA strand using a Pierce RNA 3′ End Desthiobiotinylatation Kit (Thermo, 20163). An RNA Pull-Down Kit (Thermo, 20146) was then used to detect the interaction between the TDP-43 protein and each fragment. Briefly, biotin-labeled RNAs were incubated with Hc11 cellular extract for 1 h at 4 °C. The RNA-protein complexes were then washed three times with wash buffer, followed by protein elution and western blot analysis.

**RNA immunoprecipitation.** The RIP Kit (Millipore Cat. #17-701) was used for RNA immunoprecipitation assay according to the manufacturer’s instructions. The MECS or differentiated HC1 cells were collected and lysed in RIP lysis buffer. The cell lysate was then immunoprecipitated with TDP-43 (1:100) (Proteintech, 10782-2-AP), Flag (1:5000) (CST, #14793), or IgG (5 μg) in the absence of 1 μg of NS2B/NS3 (Millipore, #CM00621), and protein A/G magnetic beads for 6–8 h at 4 °C. The beads were washed with RIP buffer, followed by RNA purification and qRT-PCR analysis.

**Mouse milk collection and analysis.** Female mice were separated from their pups for 3–5 h at 12 or 110, anesthetized with xylazine at a dose of 10 mg/kg, and injected intraperitoneally with 10 or 0.2 U of oxytocin (Sigma) to induce milk letdown, as suggested by Wang et al. and Boxer et al. After 10 min, milk was collected from the mammary glands. Milk collection was performed according to previously reported methods, with minor modification. One researcher held the anesthetized mouse while manually expressing the milk, and another researcher collected the milk using a P-200 Pipetman into a 1.5-ml tube. Each milk drop was washed with RIP buffer, followed by RNA puriﬁcation and qRT-PCR analysis.

**TAG quantification and gas chromatography analysis.** The TAG content in intracellular MECS was determined using a Triglyceride Assay Kit (Applygen Technologies, E1013) according to the manufacturer’s protocols. The TAG levels of each sample were normalized to the protein concentration measured by a BCA Protein Assay Kit. The fatty acid composition of milk was analyzed following published methods, with minor modification. In brief, 20 ml of milk was extracted with 1 ml of MeOH (containing 2.5% H2O2, 0.5% Na2S2O4), then added with 1 ml of chloroform and 1 ml of water and shaken vigorously. The solution was then centrifuged at 2500 × g for 1 min at room temperature. The super phase containing fatty acid methyl esters (FAMEs) was removed into another tube, with 2 μl of FAMEs then injected for gas chromatography (Agilent 7890A) analysis.

**Electron microscopy.** Procedures for electron microscopy were performed according to previous protocols, with minor modification. The fourth mammary gland was removed with 95% ethanol/acetone for 1 h at 4 °C, followed by serial ethanol dehydration and embedding in Epon 812. Thin sections were then stained with a 1% aqueous solution of uranyl acetate for 60 min in an ultrasonic processor. Ultrathin sections were then loaded onto 100-mesh Cu grids and double-stained with 2% uranyl acetate and lead citrate before observations employing a JEM 1400 Plus transmission electron microscope at 120 kV.

**RNA-seq analysis.** Raw sequence data were processed through standard Illumina pipelines for base-calling and fastq file generation. Paired-end reads were mapped to the mouse genome primary assembly (NCBI37) and the Ensemble mouse gene annotation for NCBI37 genebuild was used to improve mapping accuracy with STAR v2.4.2a. FeatureCounts v1.4.6-p5 was used to assign sequence reads to genes. Mitochondrial genes, ribosomal genes, and genes possessing less than five raw reads in half the samples were removed. Differential expression analysis was performed with the Bioconductor edgeR package v1.69. Significant genes were determined by an adjusted P value of <0.01 based on Benjamini-Hochberg multiple testing correction and log2-transformed fold change of >1 or <−1.

**Human milk sample analysis.** Fresh milk was obtained on days 3–5 postpartum from 60 healthy women who gave birth to full-term infants, and who exhibited similar time span of pregnancy and time of gestation. Written informed consent was obtained from all participants. In the early morning period, the donor manually pumped 3–5 ml of breast milk into a sterile, RNase-free collection tube, which was immediately placed on ice. Subsequent procedures of MFG isolation were finished within 3 h, with samples then transferred to a −80 °C freezer. We employed the RNA sampling method proposed by Maningat et al. Milk was mixed well before centrifugation at 1000 × g for 10 min at 4 °C. The lipid fraction containing MFGs in the upper phase was transferred to another 2-ml RNase-free tube. The MFGs were washed with cold PBS, and then 1.5 ml of TRIzol lysis reagent was added to the lipid fraction for RNA extraction according to the manufacturer’s instructions. RNA quality was checked by 2% agarose gel electrophoresis. A questionnaire follow-up by telephone interview was performed during the 7–8-week lactation period, which confirmed that partial breastfeeding and formula-feeding mothers were driven by necessity. The Ethics Committee of the Third Affiliated Hospital of Chongqing Medical University approved the project.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The RNA-seq data were deposited in the NCBI GEO database under ID code: GSE116456. The data that support our findings in this study are available from the corresponding author on reasonable request. The data supporting this publication includes: the Nature Research Reporting Summary linked to this article. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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**Acknowledgements**
This work was equally supported by the National Key Research and Development Program of China (Grant No. 2016YFA0109000), Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB13030400), National Science Foundation of China (Grant Nos. 31970612, U1802285, 81902714, 31801249), Yunnan Fundamental Research Projects (Grant Nos. 2018FA002, 2015HA026), Open Project from the State Key Laboratory of Genetic Resources and Evolution (Grant No. GREKF16-13 to L.A.). We thank Xun Huang, Bin Liang, Peng Shi, Ceshi Chen, and Wen Wang for constructive suggestions and Christine Watts for English editing. We would like to thank the Kunming Biological Diversity Regional Center of Instruments, Kunming Institute of Zoology, Chinese Academy of Sciences for our Electron Microscopy work and we would be grateful to Yingqi Guo for her help of making EM sample and taking images. We would like to thank Jieyu Wu and Xue Jiang for help with gas chromatography analysis.

**Author contributions**
L.Z., H.K. and B.J. designed the experiments, interpreted the results, and wrote the manuscript. L.Z. and H.K. performed the experiments. G.-D.W. and L.W. performed the evolutionary analysis. P.Y., S.X., M.L. and M.Z. collected clinical milk samples. H.X. and H.Z. analyzed the RNA-seq data. L.A., L.L., Q.Y. and L.Z. provided experimental assistance. C.-K.J.S. provided the *Tardbp* conditional floxed mice and discussed the results.

**Competing interests**
The authors declare no competing interests.

**Additional information**
*Supplementary information* is available for this paper at https://doi.org/10.1038/s41467-019-14183-1.

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**Peer review information** *Nature Communications* thanks Ian Mather, Michel Vervoort, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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