Serial section immunogold electron microscopy of phosphorylated Connexin 43 in ovarian granulosa cells

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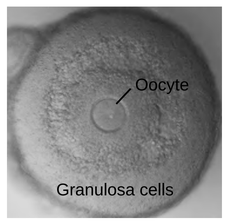
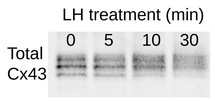
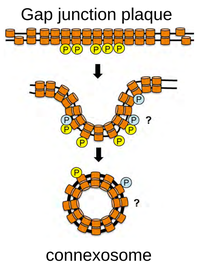
INTRODUCTION

Mammalian ovarian follicles comprise a centrally located oocyte surrounded by somatic cells that are connected to each other by gap junctions made of Connexin 43 (Cx43). These junctions are required for ovarian follicle development and for meiotic arrest of oocytes. When meiotic resumption is stimulated with luteinizing hormone (LH), Cx43 phosphorylation increases on MAP kinase sites (Norris et al., 2008), and more internalized gap junctions (connexosomes) are observed in ovarian granulosa cells (Larsen et al., 1987).

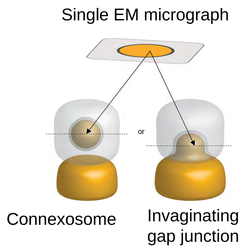






Connexosome formation involves phosphorylation of the C-terminal connexin tail (Solan and Lampe, 2016), however, specifically phosphorylated Cx43 has not been localized at the resolution needed to discern invaginating gap junctions from connexosomes.

Serial section electron microscopy (EM) combined with Immuno EM is amenable to this problem (Norris et al., 2017), and it is now easier to collect and image large numbers of serial tissue sections by using an automatic tape collecting ultramicrotome and scanning electron microscopy (Terasaki et al., 2013; Kasthuri et al, 2015)



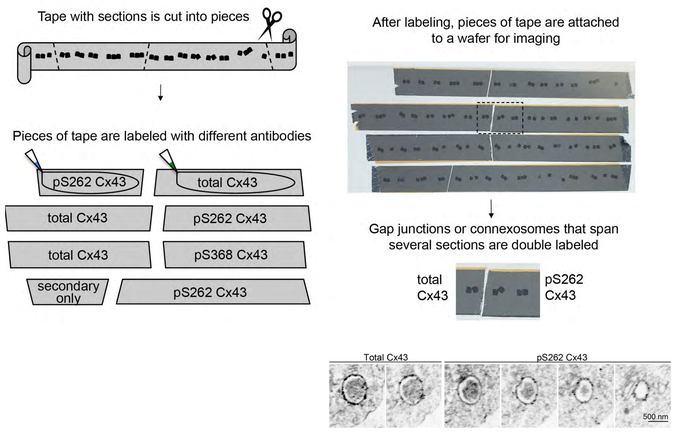
Here we investigated the 3D ultrastructural localization of specifically phosphorylated Cx43 in ovarian follicles before and during meiotic resumption.

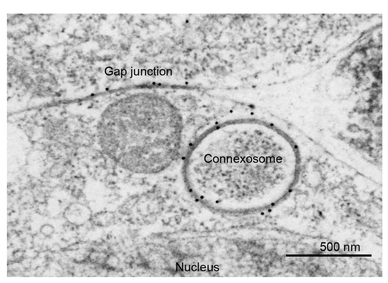
METHODS

Collect and culture ovarian follicles before or after stimulating meiotic resumption and process for Immuno EM.

- High pressure freeze
- Freeze substitute with 1.5% Uranyl Acetate in Methanol
- Embed in Lowicryl HM20

- o Collect 60 nm serial sections with an automatic tape collector and ultramicrotome (ATUM) (Kasthuri et al., 2015).
- o For ImmunoEM, label sections with Cx43 antibodies followed by 10 nm gold conjugated secondary antibody. See scheme for multi-labeling. Post-stain with Uranyl acetate.
- o Attach sections on tape to a silicon wafer, carbon coat, then image with SEM, using a backscatter detector.





A Cx43 antibody labels gap junctions and connexosomes. Connexosomes are recognizable by a thick, double membrane, with a lucid area next to the inner membrane.

RESULTS- FIGURE 1

Serial sections reveal if a ring-shaped gap junction in an ovarian granulosa cell is a connexosome (A) or invaginating gap junction (B).

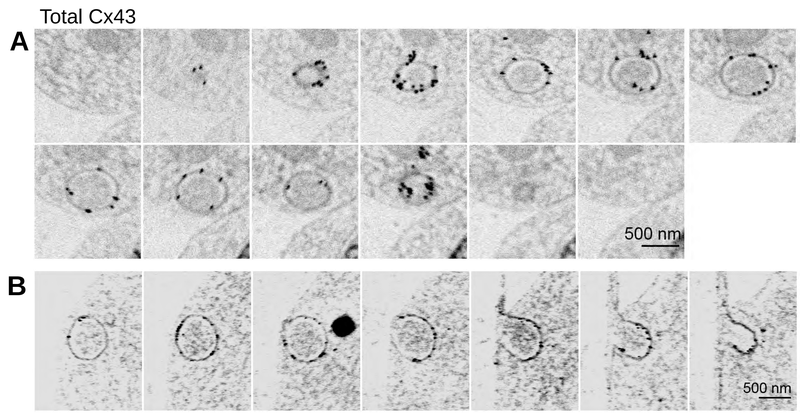
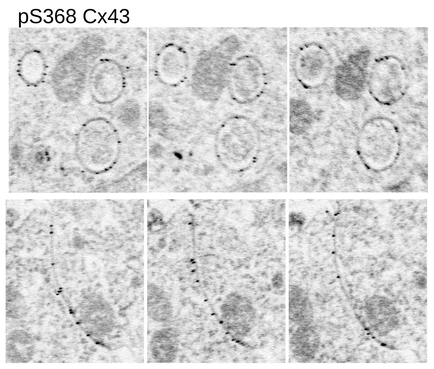
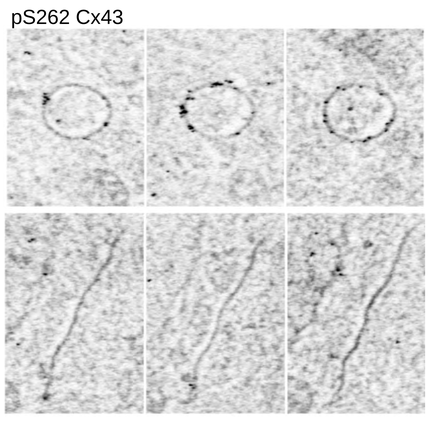


FIGURE 2



In preovulatory follicles before LH treatment, Connexin 43 is phosphorylated on S368 in both connexosomes (top) and gap junctions (bottom).

FIGURE 3



Connexin 43 is phosphorylated on S262 only in some connexosomes but not in gap junctions.

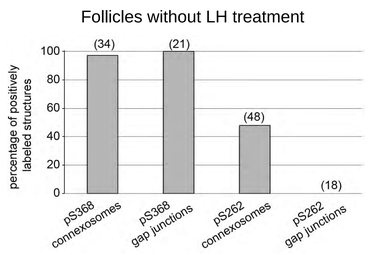


FIGURE 4

In LH treated ovarian follicles, Cx43 phosphorylation patterns change. Cx43 in both gap junctions and connexosomes has less phosphorylation on S368, while Cx43 in some gap junctions is phosphorylated on S262.

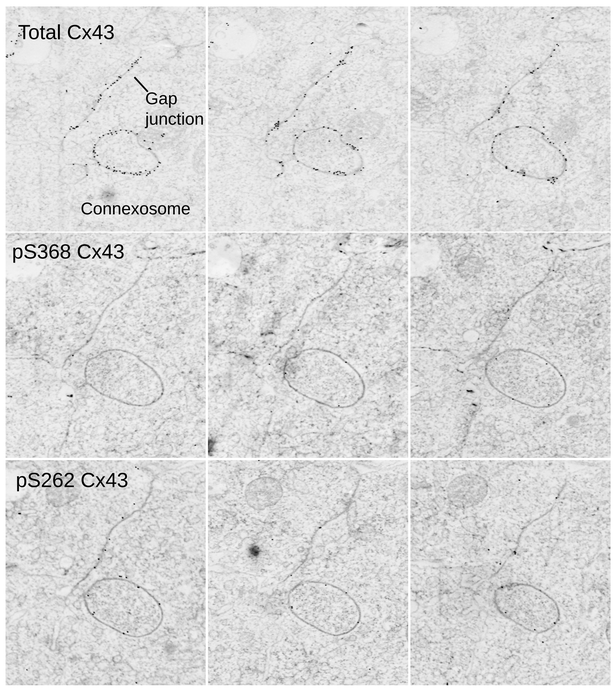


FIGURE 5

Connexosomes are often in clusters and can associate with other organelles.

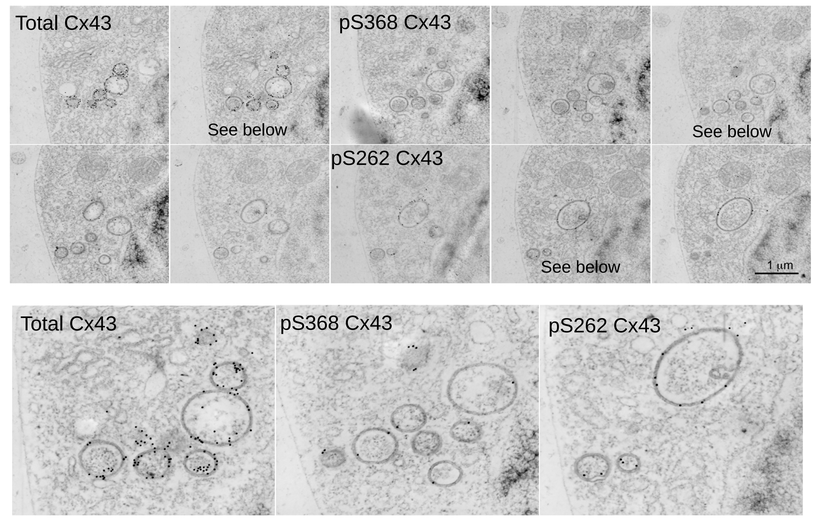
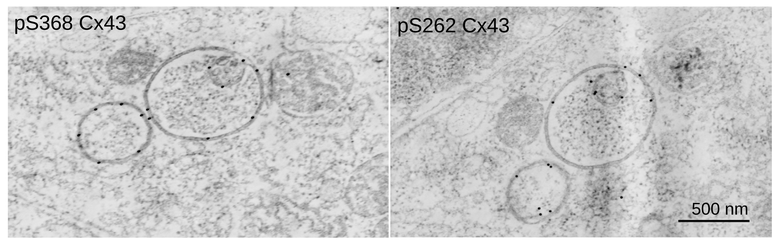
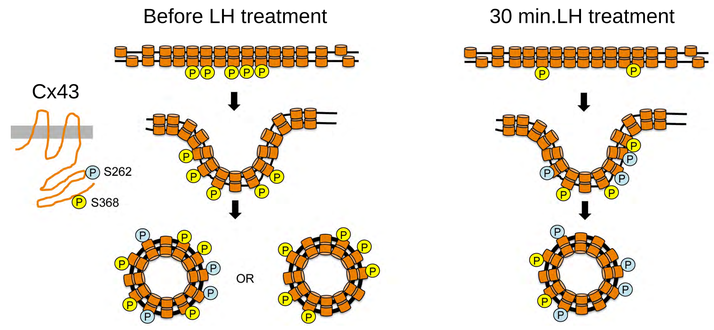


FIGURE 6

Cx43 remains phosphorylated on S368 and S262 when connexosomes undergo processing before degradation.



Summary



- Serial section immuno-EM allows us to investigate protein localization at a three-dimensional, ultrastructural level. With a post-embedding approach, we can multiplex labeling of gap junctions and connexosomes.
- Results detected by Western blot can be investigated in intact tissues to determine precisely where phosphorylated Cx43 is localized.

Future Directions

- What factors determine the direction of GJ internalization?
- Do gap junction vesicles in ovarian cells play a role in intracellular functions?

References

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