Dataset Brief

Arrestin-domain containing protein 1 (Arrdc1) regulates the protein cargo and release of extracellular vesicles

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Abbreviations:

EVs: Extracellular vesicles

Rsc: Relative spectral count

TEM: Transmission electron microscopy

NTA: Nanoparticle tracking analysis

MEFs: Mouse embryonic fibroblasts

Arrdc: Arrestin-domain containing proteins

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Abstract

Extracellular vesicles (EVs) are lipid-bilayered vesicles that are released by multiple cell types and contain nucleic acids and proteins. Very little is known about how the cargo is packaged into EVs. Ubiquitination of proteins is a key post-translational modification that regulates protein stability and trafficking to subcellular compartments including EVs. Recently, Arrdc1, an adaptor for the Nedd4 family of ubiquitin ligases, has been implicated in the release of ectosomes, a subtype of EV that bud from the plasma membrane. However, it is currently unknown whether Arrdc1 can regulate the release of exosomes, a class of EVs that are derived endocytically. Furthermore, it is unclear whether Arrdc1 can regulate the sorting of protein cargo into the EVs. In this study, exosomes and ectosomes were isolated from mouse embryonic fibroblasts isolated from wild type and Arrdc1-deficient ($Arrdc1^{-/-}$) mice. NTA based EV quantitation showed that Arrdc1 regulates the release of both exosomes and ectosomes. Proteomic analysis highlighted the change in protein cargo in EVs upon deletion of Arrdc1. Functional enrichment analysis revealed the enrichment of mitochondrial proteins in ectosomes, while proteins implicated in apoptotic cleavage of cell adhesion proteins and formation of cornified envelope were significantly depleted in exosomes upon knockout of Arrdc1.

Protein ubiquitination is a reversible post-translational modification that regulates protein degradation, stabilization, sorting and subcellular localization [1, 2]. Aberrant ubiquitination has been implicated in various pathological conditions including cancer, neurodegenerative disorders, metabolic and inflammatory diseases [3]. The ubiquitin ligases (E3s) determine the substrate specificity of ubiquitination by binding to target proteins directly or through accessory or adaptor proteins. Among the adaptor proteins, arrestin-domain containing proteins (Arrdc) facilitate the recruitment of the Nedd4 family of E3s to their substrates at the plasma membrane [4]. Emerging evidence suggests that ubiquitination is critical for the biogenesis of extracellular vesicles (EV) and cargo sorting [5-7]. EVs are a class of membranous vesicles that are released by various cell types [8]. EVs can be classified as exosomes that originate from the endosomes, ectosomes or shed microvesicles that bud directly from the plasma membrane and apoptotic bodies [9, 10]. As EVs carry nucleic acids and proteins, they are considered as mediators of intercellular communication [11, 12]. The physiological importance of EVs can be further justified by their abundance in various bodily fluids including blood, milk, saliva, urine and cerebrospinal fluid [10]. Recently, Arrdc1 has been implicated in the biogenesis and budding of microvesicles shed from the plasma membrane [7]. Furthermore, both Arrdc1 and Arrdc4 are considered as non-redundant positive regulators of EV release [13]. However, very little is known about whether Arrdc proteins can regulate the biogenesis and release of exosomes. In addition, it is unclear whether Arrdc proteins can facilitate the sorting of protein cargo to exosomes.

In this study, we examined the proteomic cargo of EVs (exosomes and microvesicles/ectosomes) secreted by mouse embryonic fibroblasts (MEFs) derived from wild type (WT) and *Arrdc1*$^{-/-}$ (KO) mice. Prior to the isolation of EVs, the MEFs were characterised by qPCR for the expression of *Arrdc1* (**Figure 1A**). Next, immunoblotting revealed no reduction of ubiquitinated proteins in the whole cell lysates (WCL) upon loss of Arrdc1 (**Figure 1B**). This lack of change in the ubiquitinated proteins could be due to the functional redundancy among the structurally similar Arrdc family proteins [14]. As Arrdc1 mostly performs as an adaptor protein for the Nedd4 family of E3s, immunoblotting was performed for Nedd4 and Nedd4-2. No significant difference was observed between the WT and KO WCL for the protein expression of Nedd4 and Nedd4-2 (**Figure 1B**). Next, EVs were isolated from the conditioned media of WT and *Arrdc1*$^{-/-}$ MEFs by differential centrifugation

coupled with ultracentrifugation. The pellet obtained after 10,000 *g* was referred to as ectosomes while the 100,000 *g* pellet was referred to as exosomes. From WT MEFs, Nanoparticle tracking analysis (NTA) of EVs revealed an average peak density of 158 and 175 nm for exosomes and ectosomes, respectively (**Figure 1C**). Similarly, EVs from *Arrdc1*$^{-/-}$ MEFs had an average peak density of 149 and 168 nm for exosomes and ectosomes, respectively (**Figure 1C**). Consistent with our previous observations [13], *Arrdc1* KO resulted in a reduction in the amount of EVs secreted (**Figure 1C-E**). Transmission electron microscopy confirmed the presence of EVs and similar shape could be observed between WT and *Arrdc1*$^{-/-}$ cell-derived EVs (**Figure 1F**). Immunoblotting confirmed the high abundance of EV enriched protein Alix in exosomes and ectosomes but could not be detected in WCL (**Figure 2A**). However, Tsg101 was detected in the WCL of both WT and *Arrdc1*$^{-/-}$ MEFs and could be detected in lower abundance in the EVs. CD9 was detected in similar abundance in the WCL, ectosomes and exosomes. Consistent with previous reports [15, 16], Alix and Tsg101 were enriched in exosomes when compared to ectosomes. Ectosomal enriched protein MMP2 [15, 17], was detected in similar abundance in both exosomes and ectosomes. This data suggests that cell-type independent ectosomal markers or enriched proteins in ectosomes are currently lacking.

Next, equal protein amount (30 µg) of the WCL, ectosomes and exosomes fraction of WT and *Arrdc1*$^{-/-}$ MEFs were subjected to label-free quantitative proteomics analysis as described previously [18, 19]. Briefly, proteins were separated by 1D-SDS PAGE, gel bands were excised, reduced, alkylated and subjected to trypsin digestion. The extracted peptides were analysed by a Q-Exactive plus mass spectrometer. The resulting MS/MS spectra was searched using X!Tandem against mouse RefSeq protein database. A total of 4,158 proteins were identified with an FDR of <1% (**Supporting Table 1**). As shown in **Figure 2B**, the proteomic profile of the WCL, ectosomes and exosomes were clustered via heatmap using FunRich [20]. The WT and the KO fractions clustered together for WCL, ectosomes and exosomes. However, compared to WCL, the proteomic profiles of ectosomes and exosomes were closer as highlighted by the dendrogram. Among the 4,158 proteins, differentially abundant proteins (>2-fold) were considered further for quantitative Venn diagram and enrichment analysis. As expected, loss of Arrdc1 resulted in the enrichment of 26 proteins and depletion of 101 proteins in MEF WCL (**Figure 2C**). Pathway analysis of the 101 depleted proteins in WCL upon loss of Arrdc1 revealed enrichment of proteins implicated in

apoptotic cleavage of cell adhesion proteins, depolymerisation of nuclear lamina and formation of cornified envelope (**Supporting Information Figure 1A**). Similarly, a Venn diagram for proteins differentially abundant in ectosomes highlighted the enrichment of 70 proteins in *Arrdc1*$^{-/-}$ MEF-derived ectosomes (**Figure 2D**). A total of 73 proteins were depleted in ectosomes due to the loss of Arrdc1. However, 93 proteins were depleted in exosomes upon loss of Arrdc1. Interestingly, a total of 93 proteins were enriched in exosomes secreted by *Arrdc1*$^{-/-}$ cells. When proteins detected in ectosomes and exosomes were subjected to cellular component based enrichment analysis, proteins resident to mitochondrion were enriched in ectosomes while the terms extracellular exosome, plasma membrane and cytoplasm were enriched in exosomes (**Supporting Information Figure 1B**). The top 20 abundant proteins detected in exosomes secreted by *Arrdc1*$^{-/-}$ cells include Dock5, Pebp1, Fzd7, Lrp1 and Ldhc (**Supporting Information Figure 1C**).

Next, proteins that are in high abundance in ectosomes secreted by WT and *Arrdc1*$^{-/-}$ cells were analysed for pathway enrichment using FunRich. Ectosomes from *Arrdc1*$^{-/-}$ cells were depleted in proteins implicated in urea cycle and transport between ER and Golgi memebrane (**Figure 3A**). On the contrary, proteins implicated in PTK6 regulates cell cycle, transcriptional regulation by RUNX2 and transcription of DNA repair genes were significantly enriched in ectosomes secreted by *Arrdc1*$^{-/-}$ cells. High abundant proteins in exosomes secreted by WT and *Arrdc1*$^{-/-}$ cells were also analysed for pathway enrichment using FunRich. Interestingly, similar to the WCL, exosomes from WT cells were significantly enriched with proteins implicated in apoptotic cleavage of cell adhesion proteins and formation of cornified envelope (**Figure 3B**). Upon Arrdc1 KO, proteins implicated in ABC-family proteins mediated transport were significantly enriched in exosomes. These results clearly demonstrate that Arrdc1 can alter the proteomic cargo of exosomes and ectosomes.

Comparing the differentially abundant proteins in ectosomes and exosomes in the context of subcellular compartment revealed the enrichment of cytosol and nuclear proteins in ectosomes compared to exosomes (**Figure 3C**). In addition, extracellular exosome proteins were enriched in exosomes compared to ectosomes. Whilst this observation may provide clues on key signaling attributes between these EVs, the enrichment partly could be attributed to the surface area of the EVs. To validate the change in cargo by immunoblotting, membrane proteins in EVs were shortlisted based on higher abundance. Fzd7 was 3.5-fold more

abundant in exosomes secreted by *Arrdc1*^{-/-} cells. Importantly, Fzd7 abundance did not change in the WCL or ectosomes but was significantly enriched in exosomes secreted by *Arrdc1*^{-/-} cells (P=0.02). Fzd7 is a seven transmembrane domain containing protein that is implicated in Wnt signaling, metastasis and pluripotency [21, 22]. Similarly, Dock5 (4-fold) and Pebp1 (4-fold) were enriched only in exosomes secreted by *Arrdc1*^{-/-} cells but were detected at similar abundance in WCL of WT and *Arrdc1*^{-/-} cells. These observations suggest that sorting of protein cargo into exosomes is dependent on Arrdc1. In addition, Fat1, a protocadherin implicated in epithelial-to-mesenchymal transition and in the negative regulation of Wnt signaling pathway was also abundant in the WCL and exosomes from WT MEFs. Immunoblotting confirmed the enrichment of Fzd7 in exosomes released by *Arrdc1*^{-/-} cells (**Figure 3D**). In contrast, Fat1 was found in lower abundance in WCL and exosomes derived from *Arrdc1*^{-/-} cells. However, Fat1 was not significantly differentially abundant (P=0.09) based on the proteomic data. This difference between MS and Western bot analysis can be attributed to the hydrophobic nature of peptides that are often underrepresented in MS analysis of membrane proteins such as Fat1.

In summary, EVs were isolated from WT and *Arrdc1*^{-/-} MEFs and subjected to label-free quantitative proteomics analysis. Pathway enrichment analysis of the proteomic profiles highlighted the enrichment of proteins implicated in ubiquitination and antigen presentation in exosomes secreted by WT MEFs. On the contrary, ectosomes from WT MEFs were enriched with mitochondrial proteins while ectosomes from *Arrdc1*^{-/-} MEFs were enriched with proteins implicated in meiotic recombination and DNA methylation. Though some of the enriched functional pathways overlap between exosomes and ectosomes, these results suggest that the EV subtypes may have different functions under physiological conditions. In addition, these results corroborate the role of Arrdc1 in release of EVs and protein cargo sorting into EVs. Further studies are needed to understand the redundancy of the family of arrestin-domain containing proteins in EV biogenesis, release and cargo sorting.

Acknowledgement

Conflict of interest

The authors declare no conflict of interest

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Figure legends

Figure 1

Characterization of MEFs and exosomes

(**A**) qPCR analysis for the mRNA expression of Arrdc1 is depicted in WT and *Arrdc1*$^{-/-}$ (KO) MEFs. The mRNA expression analysis confirms the deletion of Arrdc1. (**B**) Western blotting analysis of WCL (40 μg) from WT and *Arrdc1*$^{-/-}$ MEFs. Ubiquitinated proteins were not affected by the depletion of Arrdc1 as depicted by the quantitative Western blotting for ubiquitinated proteins. No significant difference in the abundance of Nedd4 or Nedd4-2 was observed. (**C**) Nanoparticle tracking analysis (NTA) of EVs obtained by differential ultracentrifugation shows the presence of particles. Equal number of cells were used in the analysis to quantify the number of particles present in the EV fractions. (**D**) The size distribution of particles in ectosomes and exosomes released by WT and *Arrdc-/-* cells as quantified by NTA. (**E**) Total number of particles released by WT and *Arrdc-/-* cells as quantified by NTA. (**F**) Transmission electron microscope images of exosomes and ectosomes confirms the presence of EVs. All data are represented as mean ± s.e.m. * denotes P < 0.05, ** denotes P<0.01 as determined by t-test.
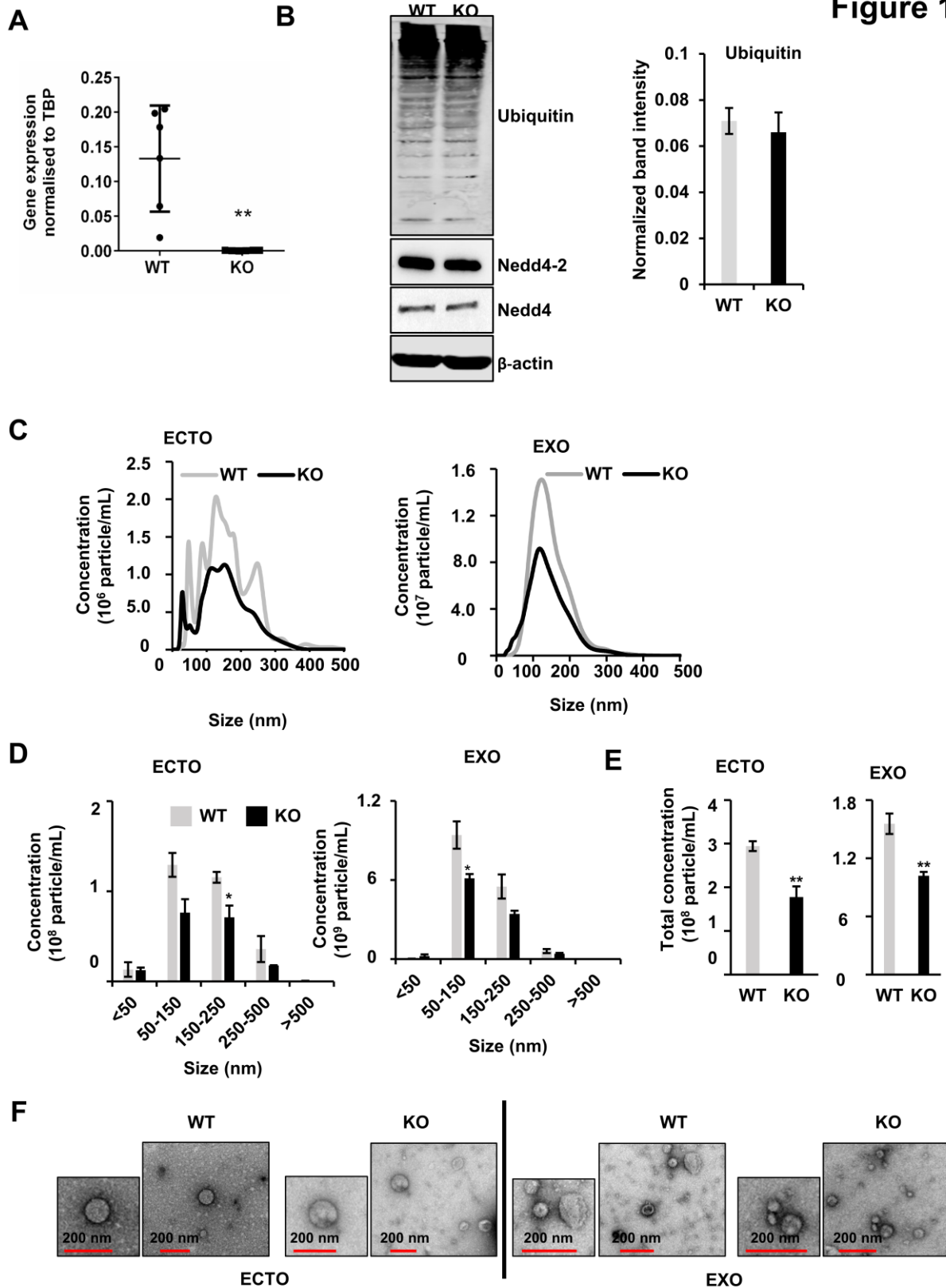
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Figure 1

Figure 2

Proteomic profile of WCL and EVs isolated from WT and Arrdc1 KO MEFs

(**A**) Immunoblotting for EV enriched proteins Alix (50 µg), Tsg101 (20 µg), CD9 (20 µg) and MMP-2 (40 µg). (**B**) FunRich based heatmap of proteins (average abundance in three biological replicates) that are differentially abundant in WCL, ectosomes and exosomes secreted by MEFs. The three fractions of WCL, ectosomes and exosomes had unique proteome content. (**C**) FunRich based Venn diagram of differentially abundant proteins identified in WCL of WT and *Arrdc1*$^{-/-}$ MEFs. Red arrow depicts proteins that are highly abundant in WCL of *Arrdc1*$^{-/-}$ MEFs while green arrow depicts proteins that are depleted in WCL of *Arrdc1*$^{-/-}$ MEFs. (**D**) Venn diagram of differentially abundant proteins identified in ectosomes released by WT and *Arrdc1*$^{-/-}$ MEFs. Red arrow depicts proteins that are highly abundant in ectosomes of *Arrdc1*$^{-/-}$ MEFs while green arrow depicts proteins that are depleted in ectosomes of *Arrdc1*$^{-/-}$ MEFs. (**E**) Venn diagram of differentially abundant proteins identified in exosomes secreted by WT and *Arrdc1*$^{-/-}$ MEFs. Red arrow depicts proteins that are highly abundant in exosomes of *Arrdc1*$^{-/-}$ MEFs while green arrow depicts proteins that are depleted in exosomes of *Arrdc1*$^{-/-}$ MEFs.

Figure 2

A

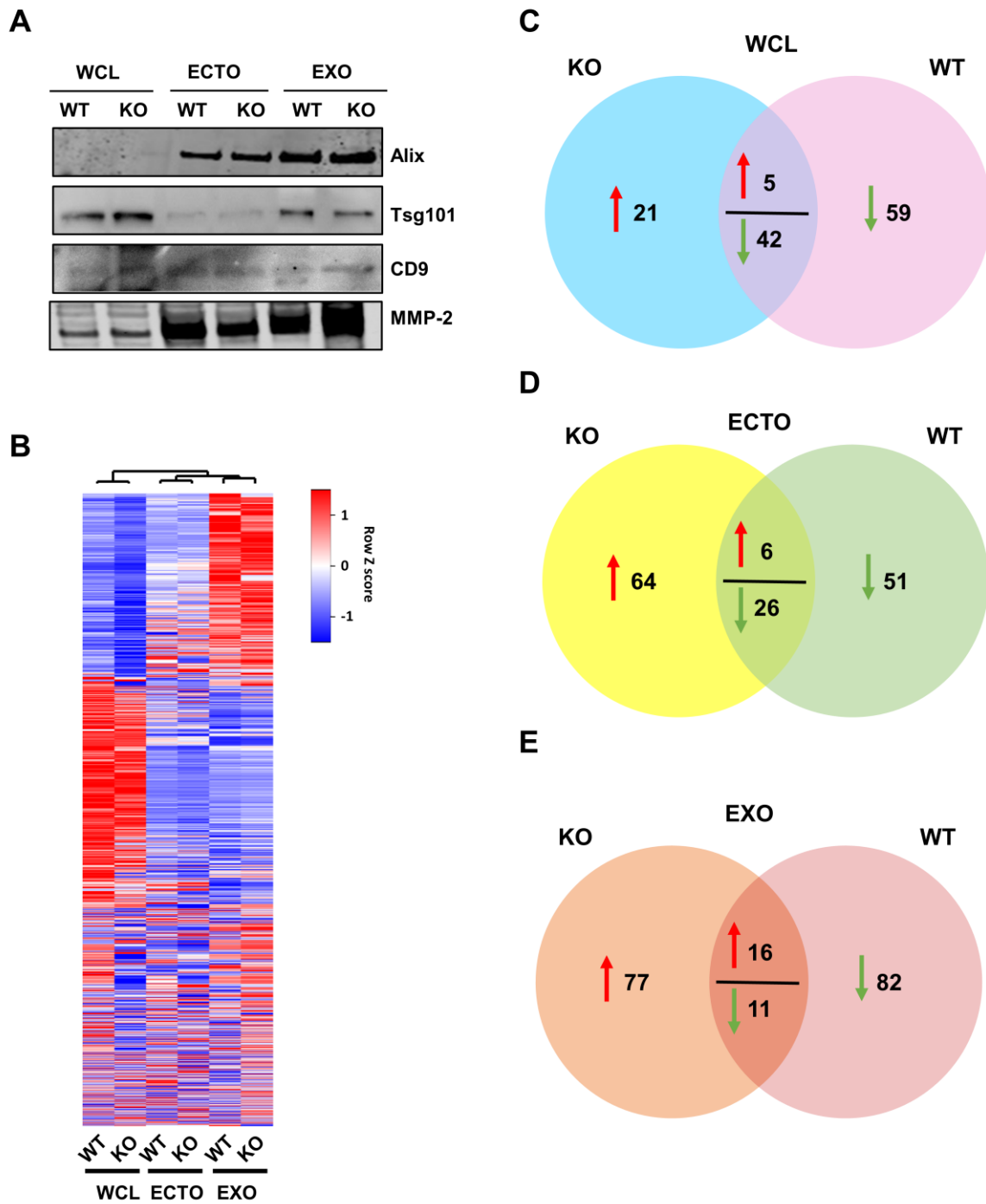| WCL	ECTO	EXO
WT KO	WT KO	WT KO

Alix

Tsg101

CD9

MMP-2

B

Row Z score

1

0

-1

WT KO WT KO WT KO

WCL ECTO EXO

C

WCL

KO   WT

↑ 5

↑ 21

↓ 42

↓ 59

D

ECTO

KO   WT

↑ 6

↑ 64

↓ 26

↓ 51

E

EXO

KO   WT

↑ 16

↑ 77

↓ 11

↓ 82

Figure 3

Pathway enrichment analysis of proteomic cargo of EVs

(**A**) FunRich-based Reactome pathway analysis of proteins highly abundant in ectosomes released by WT and *Arrdc1*$^{-/-}$ MEFs. (**B**) FunRich-based Reactome pathway analysis of proteins highly abundant in exosomes released by WT and *Arrdc1*$^{-/-}$ MEFs. (**C**) FunRich-based subcellular component analysis of proteins in exosomes and ectosomes released by WT and *Arrdc1*$^{-/-}$ MEFs. * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$ as determined by hypergeometric test using FunRich. (**D**) Validation of proteomic data by immunoblotting (40 µg WCL; 20-40 µg EV fractions) for proteins enriched in WCL and/or EVs. Quantitative Western blot data is provided for Fzd7 (*n=6*) and Fat1 (*n=3*). All data are represented as mean ± s.e.m. * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$ as determined by t-test.

Figure 3

A

B

C

D
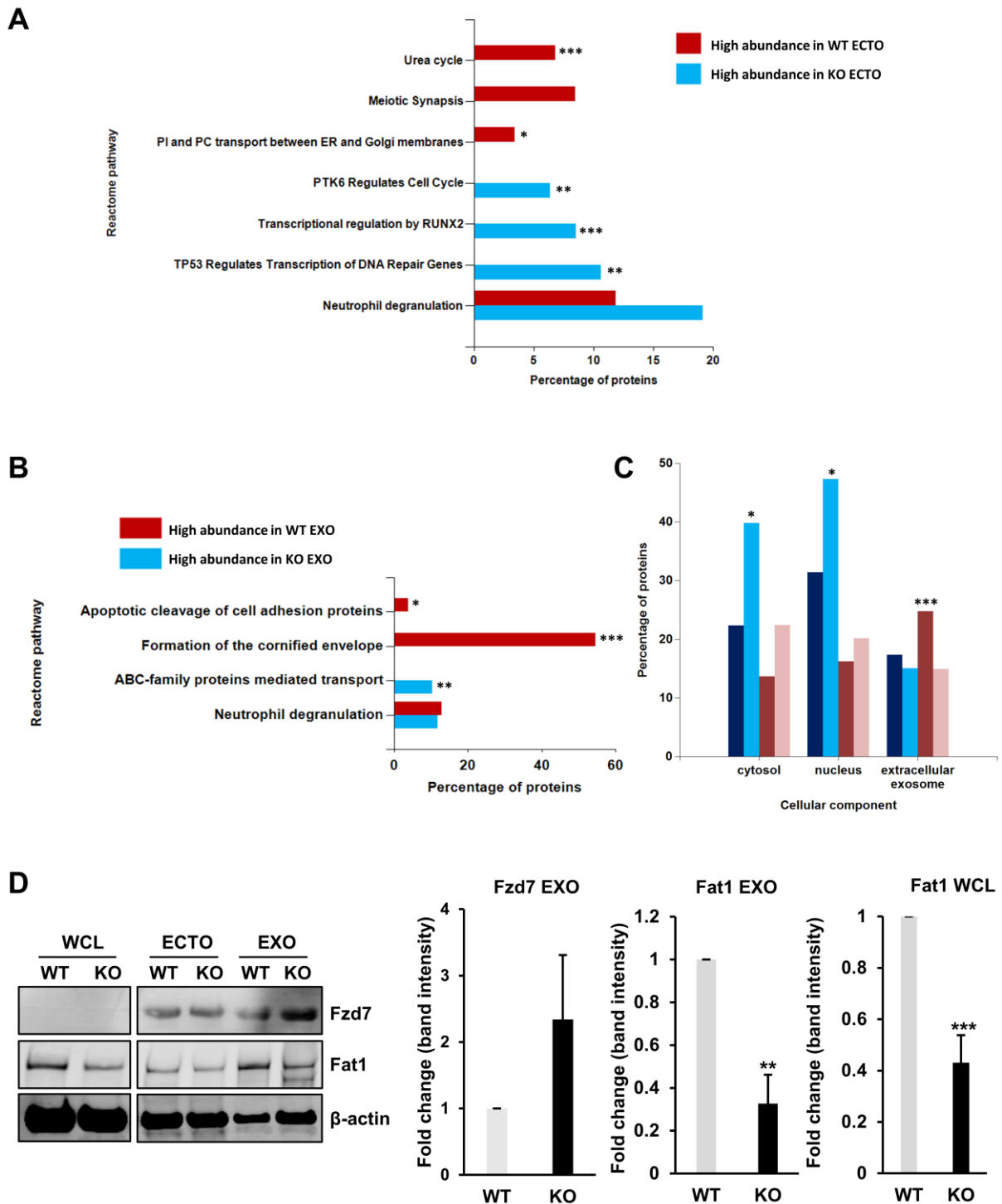
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