Supplementary Information

Tolerance to alkaline ambient pH in *Aspergillus nidulans* depends on the activity of ENA proteins.

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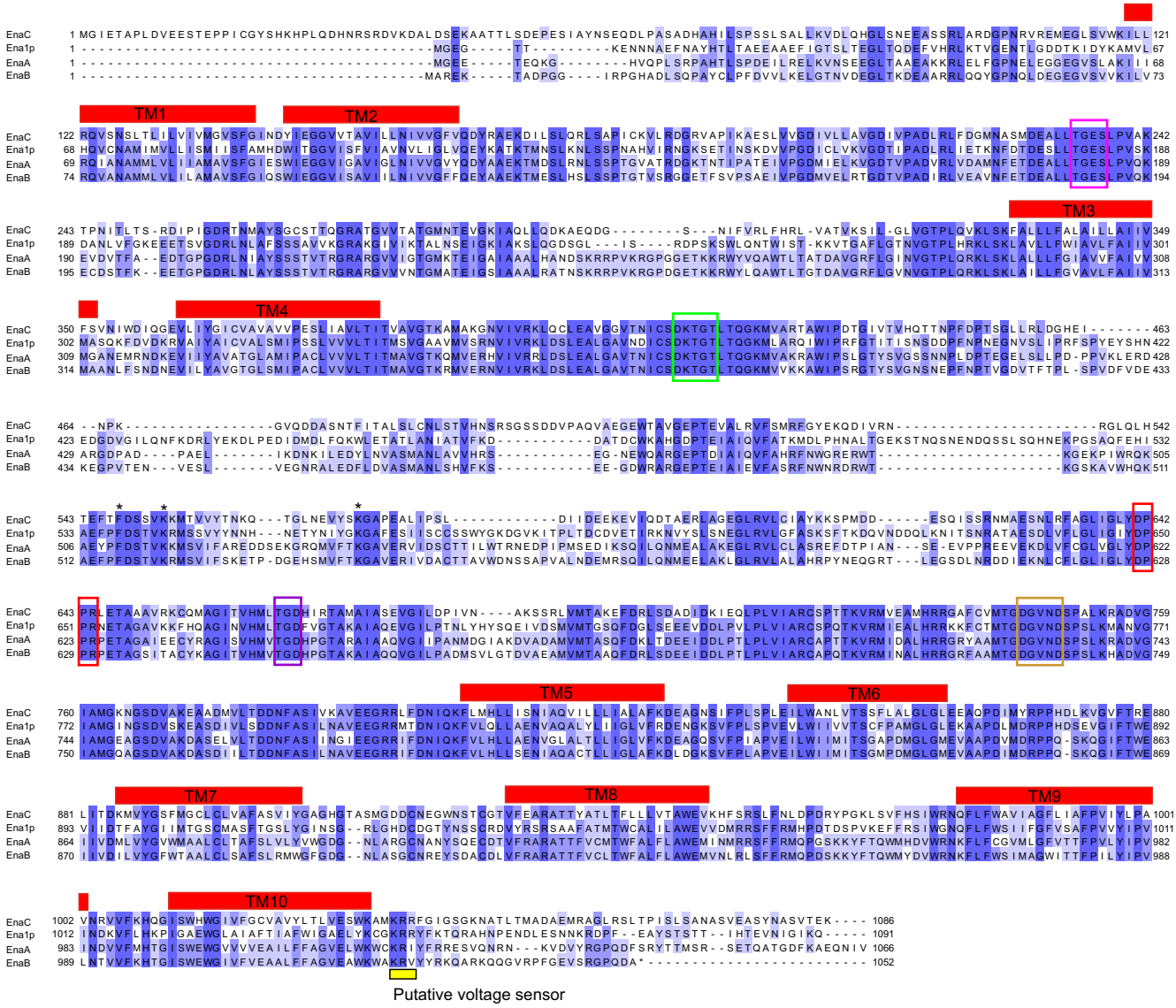
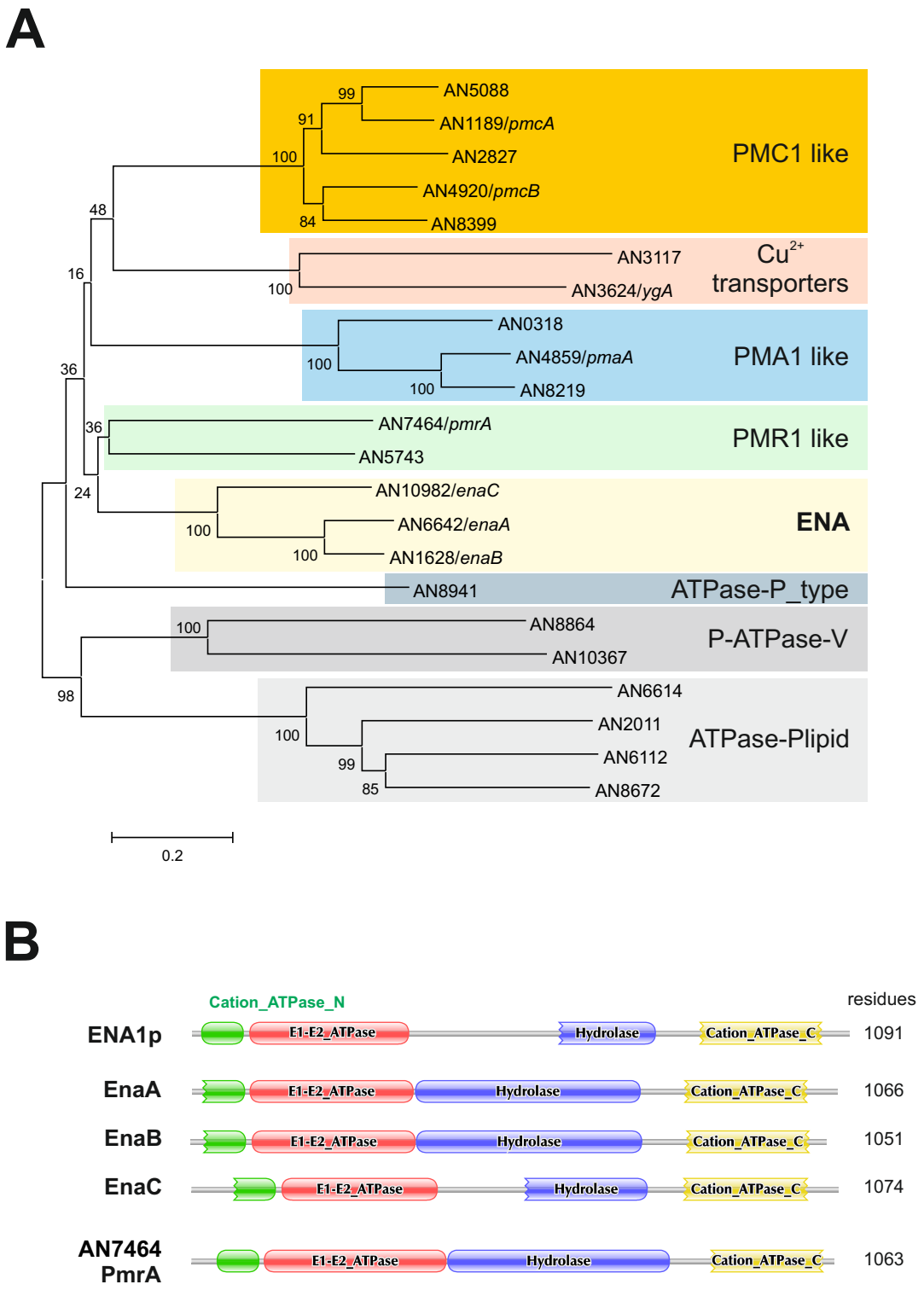
Running title: Role of P-type ATPases in alkaline pH response.



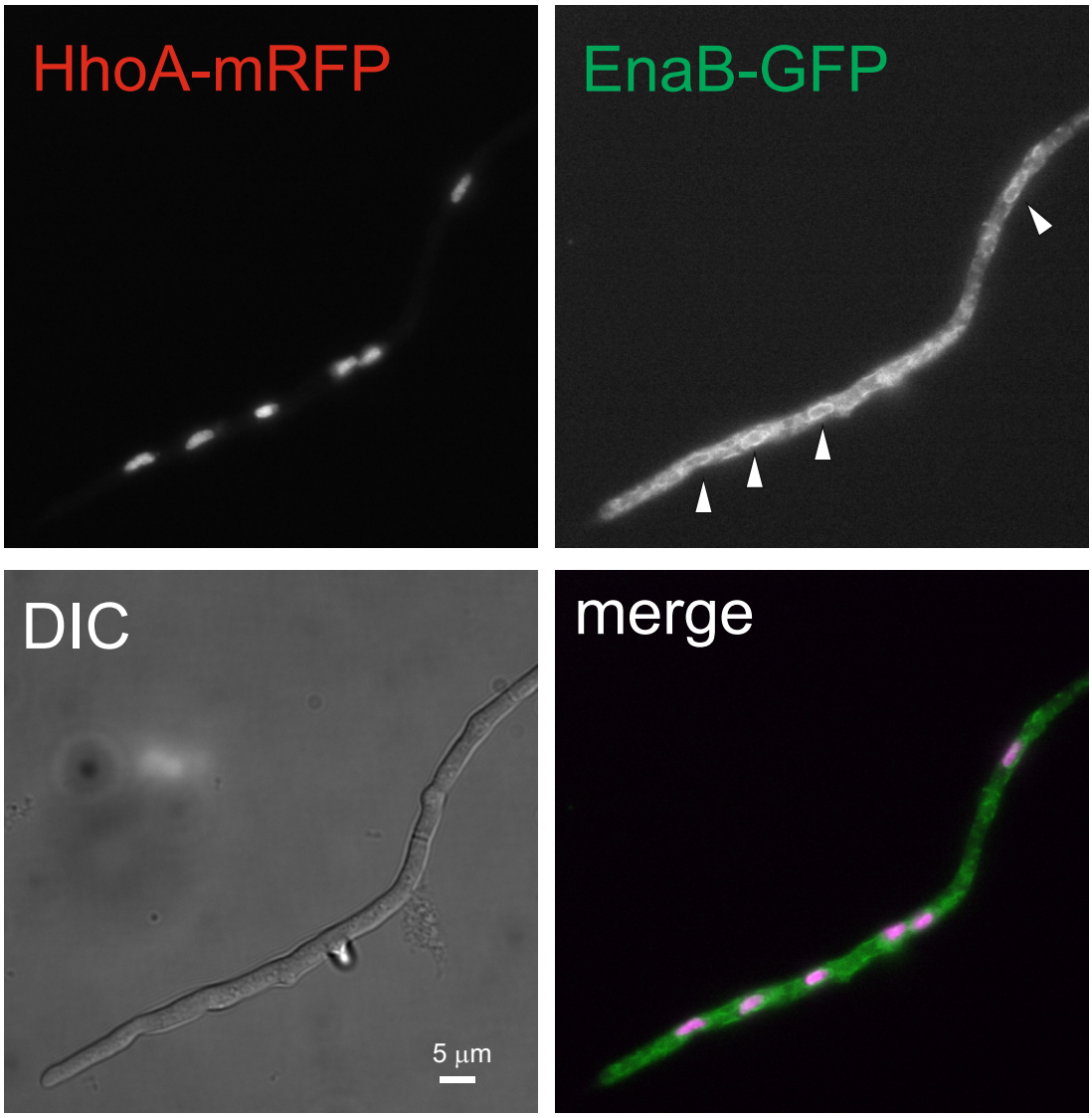
Conserved motifs for ENA proteins, coordinates as in ScEna1p

- TGES^{183} (A domain)
- DKTGT^{393} (D = Asp residue phosphorylation)
- * F^{537}, K^{542}, K^{561} (nucleotide binding)
- DPPR^{652}
- TGD^{675}
- DGVND^{761} (Mg^{2+} binding domain)

Supplementary Figure 1. Amino acid sequence analysis of An-ENA like ATPases. (**A**) Sequence alignments of *S. cerevisiae* Ena1p and *A. nidulans* predicted full-length ENA ATPases. Clustal method was used for the comparison. Boxes describe conserved functional regions detailed in reference [1] and the red rectangles delimit the position of the putative transmembrane regions predicted with Hidden Markov Models (TMHMM and HMMTOP). Protein accession numbers are reported as follows: *Saccharomyces cerevisiae* Ena1p (NP_010325), *Aspergillus nidulans* EnaA (CBF71157), EnaB (CBF85251) and EnaC (CBF79858).



Supplementary Figure 2. Classification of ATPases in *A. nidulans* and distribution of functional domains. (A) Phylogenetic tree corresponding to 22 ATPases of *A. nidulans*. The tree was generated using Mega (version 7.0)[2] and the Neighbor-Joining method, with a bootstrap test value of 10,000 replicates. EnaA, EnaB and EnaC group to the same clade of the tree, next to PMR1-like proteins. (**B**) Representation of domain organization and extension of EnaA, EnaB and EnaC according to Pfam, AspGD and FungiDB databases. Colours keyed to cation-ATPase-N (pfam00690) domain (green), E1-E2_ATPase (pfam00122) domain (red), Hydrolase, haloacid dehalogenase-like hydrolase (HAD) (COG4087. pfam00702) domain (blue), and Cation_ATPase_C (pfam00689) domain (yellow).



Supplementary Figure 3. Colocalization of EnaB-GFP and nuclei. Images showing the localization of EnaB-GFP and histone H1-mRFP in cells of diploid strain DIP5. Red fluorescence channel is shown on top left image showing a hypha with 6 nuclei. Top right is shown the green fluorescence channel (EnaB-GFP) and the ovoid structures are indicated with white arrowheads. Down-right is the merged image of red and green channels (magenta nuclei/histone H1 and green EnaB) showing the localization of EnaB at the periphery of nuclei. Down left is the Nomarsky image of the hypha and the scale bar representing 5 µm.

Supplementary Table 1. List of *A. nidulans* strains used in this work

Strain	*Genotype*	Source
MAD2446	*pyrG89; wA4; inoB2, pyroA4; hho::mCh::pyroA ;(myc)3-pacC(pacC900); veA1*	Madrid's collection
MAD2173	*pyrG89 argB2 pyroA4Dnku::argB sec63::gfp::pyrGAf,*	[3]
MAD1425	*pyrG89; argB2; pyroA4, nkuAΔ::argB; veA1*	TNO02A3 [4]
MAD1425+ /BD377	*pyrG^{An}; argB2; pyroA4, nkuAΔ::argB; veA1*	[5]
MAD1427	*pyrG89, pabaA1; argB2; nkuAΔ::argB; veA1, riboB2*	TNO02A25 [4]
MAD2732	*pyrG^{An}, pabaA1; argB2; nkuAΔ::argB; veA1, riboB2*	[5]
MAD2731	*pyrG89, pabaA1; argB2; nkuAΔ::argB; veA1, riboB^{An}*	[5]
BD604	*pyrG89, enaAΔ::pyrG^{Af}; argB2; pyroA4, nkuAΔ::argB; veA1*	This work
BD487/BER53-B	*pabaA1; argB2; nkuAΔ::argB; enaBΔ::riboB^{Af}; veA1, riboB2*	This work
BD486/BER55-B	*pyrG89, pabaA1; argB2; enaCΔ::pyrG^{Af}, ΔnkuA::argB; veA1, riboB^{An}*	This work
BD488/BER54	*pyrG89, pabaA1, enaAΔ::pyrG^{Af}; argB2; ΔnkuA::argB; enaBΔ::riboB^{Af} ; veA1, riboB2*	This work
BD489/BER56	*pyrG89, pabaA1; argB2; enaCΔ::pyrG^{Af}, ΔnkuA::argB; enaBΔ::riboB^{Af} ; veA1, riboB2*	This work
BD625	*pyrG89, enaAΔ::pyrG^{Af}; argB2; enaCΔ::pyroA^{Af}, pyroA4, nkuAΔ::argB; veA1*	This work
BD575	*pyrG89, enaAΔ::pyrG^{Af}; pyroA4, nkuAΔ::argB; enaBΔ::riboB^{Af}; inoB2; wA4, argB2*	This work
BD612	*pyrG89, enaAΔ::pyrG^{Af}; argB2; enaCΔ::pyrG^{Af}, pyroA4, nkuAΔ::argB; enaBΔ::riboB^{Af}; veA1*	This work
MAD305	*pabaA1; pacC^{c}14*	Madrid's collection
MAD1074	*inoB2; palH72*	Madrid's collection
MAD4097 /HHF27b	*sltAΔ::riboB^{f}*	[6]
MAD25ED	*pyrG89, enaA::gfp::pyrG^{Af}; pyroA4, nkuA::bar*	This work
MAD26ED	*pyrG89; pyroA4, nkuAΔ::bar; enaB::gfp::pyrG^{Af}*	This work
MAD1739	*pyrG^{An}; pyroA4, nkuAΔ::bar*	Madrid's collection
MAD3042	*pyrG89; pyroA4, nkuAΔ::bar, [pyroA+::gpdA^{mini}::enaA]*	This work
MAD3043	*pyrG89; pyroA4, nkuAΔ::bar, [pyroA+::gpdA^{mini}::enaA]x2*	This work
MAD1865	*pabaA1; pyroA4; inoB2, glrA1, palA1*	Madrid's collection
I25	*pabaA1;[pyroA+::gpdA^{mini}::enaA]; palA1*	This work
I4	*pabaA1; [pyroA+::gpdA^{mini}::enaA]x2; palA1*	This work
DIP5	*pyrG89; pyroA4, nkuAΔ::bar; enaB::gfp::pyrG^{Af} /*	This work

	pabaA1, hhoA::mRFP::pyrG^{Af}	

Supplementary Table 2. Oligonucleotides used in this work

Name	Sequence (5´-3´)	Aim	Source
EnaA-gsp1	CGGAAAGACCTTACCATGATTCGC	EnaA 5´ UTR	This work
EnaA-gsp2	CTGACGTTACTACTATGGTGTC	EnaA 5´ UTR	This work
EnaA-gsp3	GCACTCCGACGGAAAGCTTGCTACC	EnaA 3´ UTR	This work
EnaA-gsp4	CAACCACAACCTCGATACTTCGC	EnaA 3´ UTR	This work
EnaA-gsp5	TCTACGATCCGCCTAGGCCTGAGACAG C	EnaA 3´ end ORF	This work
EnaA-gsp6	AACGATATTCTGTTCCGCCTTGAAGTC TCC	EnaA 3´ end ORF	This work
EnaA-gsp2*	GACACCATAGTAGTAACGTCAGACCGG TCGCCTCAAACAATGCTCT	pyrG^{Af} cassette	This work
EnaA-gsp3*	GGTAGCAAGCTTTCCGTCGGAGTGCGT CTGAGAGGAGGCACTGATGCG	pyrG^{Af} /gfp::pyrG^{Af} cassette	This work
EnaA-gsp6*	GGAGACTTCAAGGCGGAACAGAATAT CGTTGGAGCTGGTGCAGGCGCTGGAGC C	gfp::pyrG^{Af} cassette	This work
EnaB-gsp1	CAACAACCAGCTCAGCCGTTTGC	EnaB 5´ UTR	This work
EnaB-gsp2	TGACAACTGAGATGATAAGCCCG	EnaB 5´ UTR	This work
EnaB-gsp3	CGTCTGCTTTCCCTCGCTTAG	EnaB 3´ UTR	This work
EnaB-gsp4	AGCTAGCCCTCCAAGCCAACAC	EnaB 3´ UTR	This work
EnaB-gsp5	AACTCATCGGCTCCTGTGGC	EnaB 3´ end ORF	This work
EnaB-gsp6	GGCATCCTGAGGGCCCCTGCTTACCTC	EnaB 3´ end ORF	This work
EnaB-gsp2*	CGGGCTTATCATCTCAGTTGTCAACCG GTCGCCTCAAACAATGCTCT	riboB^{Af} cassette	This work
EnaB-gsp3*	CTAAGCGAGGGAAAGCAGACGGTCTG AGAGGAGGCACTGATGCG	riboB^{Af}/gfp::pyrG^{Af} cassette	This work
EnaB-gsp6*	GAGGTAAGCAGGGGCCCTCAGGATGC CGGAGCTGGTGCAGGCGCTGGAGCC	gfp::pyrG^{Af} cassette	This work
EnaC-gsp1	GGAAAGAGTGCGGGGC	EnaC 5´ UTR (enaC-PP1)	This work
EnaC-gsp2	CATTCAATTGGAGTGGAGGTG	EnaC 5´ UTR (enaC-PP2)	This work
EnaC-gsp3	TAGGGAACGGATGTTACTTCCG	EnaC 3´ UTR	This work
EnaC-gsp4	CGCCGAGATCTCCCG	EnaC 3´ UTR	This work
EnaC-gsp2*	CACCTCCACTCCAATTGAATGCGTGGA GTTACCAGTGATTGACCAGG	riboB^{Af} / pyrG^{Af} cassette (enaC-SMP1)	This work
EnaC-gsp3*	CGGAAGTAACATCCGTTCCCTAGTCTG AGAGGAGGCACTGATGCG	riboB^{Af} / pyrG^{Af} cassette (enaC-SMP2)	This work
RiboAnid term rev	CCTAGCAGTGGTTGAATAGT	*A. nidulans* AN0670.2 Amplification	This work
RiboAnid prom fw	GATAAGTGTTGGTGGAAGTG	*A. nidulans* AN0670.2 Amplification	This work
EnaA-sense	CCTAGGCGTTGAAATCTCCC	Meiotic recombination confirmation	This work
EnaA-antis	GCTCGAAGGTGCGGG	Meiotic recombination confirmation	This work

EnaB-sense	GGCTTGACCTTCGAATCTGC	Meiotic recombination confirmation	This work
EnaB-antis	CTTACCAACAGGCCACACTCC	Meiotic recombination confirmation	This work
EnaC-sense	CGCTCCTCCTGCAATCC	Meiotic recombination confirmation	This work
EnaC-antis	CGAGCGTGTAGGCAGAGG	Meiotic recombination confirmation	This work
An6642 fw	TCAACGTCGGAACACCTCTT	*enaA* forward primer for probe	[7]
An6642 rev	TTGACACCGTCACCAGTCAT A	*enaA* reverse primer for probe	[7]
An1628 fw	TCGTCGTTGCAGGTGCTGAT	*enaB* forward primer for probe	[7]
An1628 rev	TATTGGTCACCGCTCCAAGG	*enaB* reverse primer for probe	[7]
An10982 fw	AGGACATCCTCTCGCTACAG	*enaC* forward primer for probe	[7]
An10982 rev	TCCTTCTCCAGCCAGTCTCT	*enaC* reverse primer for probe	[7]
msnA fw	CGACCATCATCTAATGTGAGTGC	*msnA* forward primer for probe	This work
msnA rev	GAGACCAGTGTGGATGTCGAAGC	*msnA* reverse primer for probe	This work
EnaAup	CGGAATTCATGGGAGAGGAGACCGAA C	*enaA* forward primer with EcoRI site for *gpda::enaA* construction	This work
EnaAdown	CGGAATTCTCAAACGATATTCTGTTCC	*enaA* reverse primer with EcoRI site for *gpda::enaA* construction	This work

Supplementary Table 3. List of plasmids used in this work

Plasmid ID Madrid's collection	Main features	Reference
p1439	Plasmid pFNO3 containing the insert GA_5-GFP-*pyrG*^{Af}	[8]
p1547	pBluescriptSK(+) derivative containing *A fumigatus pyroA* gene	Gift from B. Oakley
p1548	pBluescriptSK(+) derivative containing *A fumigatus riboB* gene	Gift from B. Oakley
p1660	Expression vector, harbouring *gpdA* mini promoter, used for moderate expression of target genes. Directed integration to *pyroA4* mutant allele.	[9]

References

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3. Markina-Iñarrairaegui, A., Pantazopoulou, A., Espeso, E.A., & Peñalva, M.A. The *Aspergillus nidulans* peripheral ER: disorganization by ER stress and persistence during mitosis. PLoS. One. **8**, e67154 (2013).

4. Nayak, T., *et al.* A versatile and efficient gene-targeting system for *Aspergillus nidulans*. Genetics **172**, 1557-1566 (2006).

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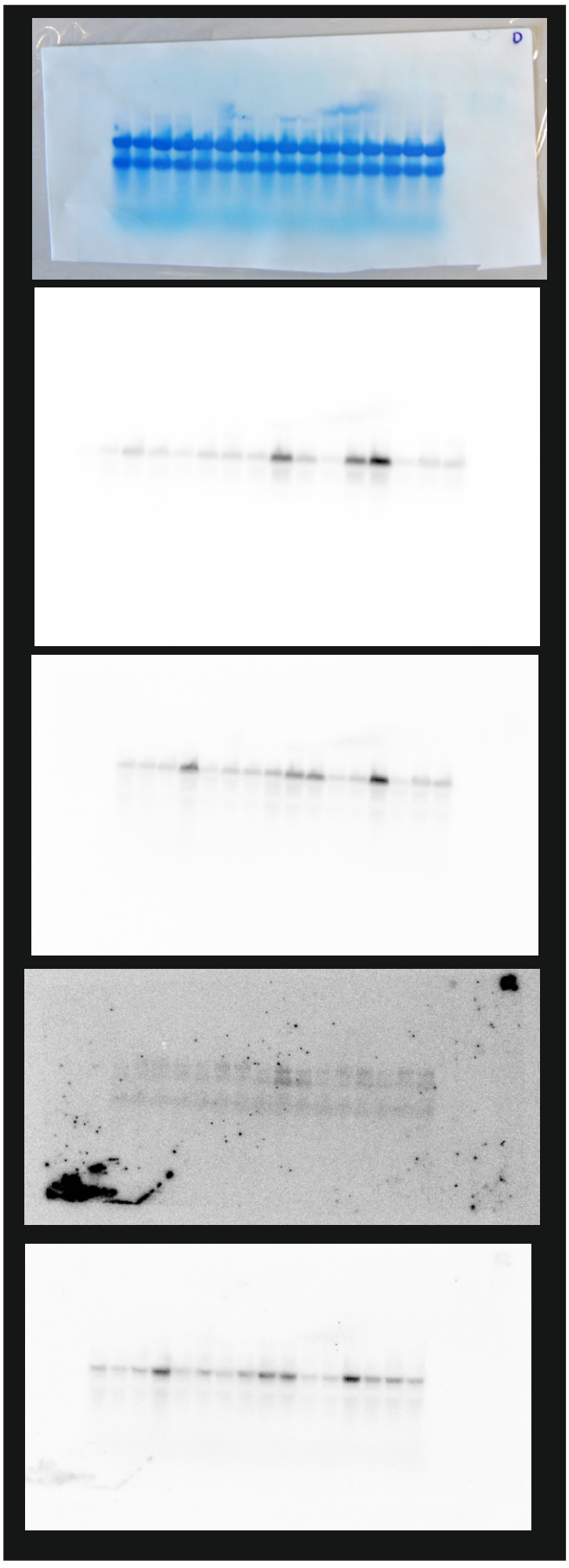
6. Findon, H., *et al.* Analysis of a novel calcium auxotrophy in *Aspergillus nidulans*. Fungal. Genet. Biol. **47**, 647-655 (2010).

7. Spielvogel, A., *et al.* Two zinc finger transcription factors, CrzA and SltA, are involved in cation homoeostasis and detoxification in *Aspergillus nidulans*. Biochem. J. **414**, 419-429 (2008).

8. Yang, L., *et al.* Rapid production of gene replacement constructs and generation of a green fluorescent protein-tagged centromeric marker in *Aspergillus nidulans*. Eukaryot. Cell **3**, 1359-1362 (2004).

9. Pantazopoulou, A. & Peñalva, M.A. Organization and dynamics of the *Aspergillus nidulans* Golgi during apical extension and mitosis. Mol. Biol. Cell **20**, 4335-4347 (2009).

Full size images for hybridations of wild type samples filter
See Figure 3 panel A and legend for lanes information.



rRNA

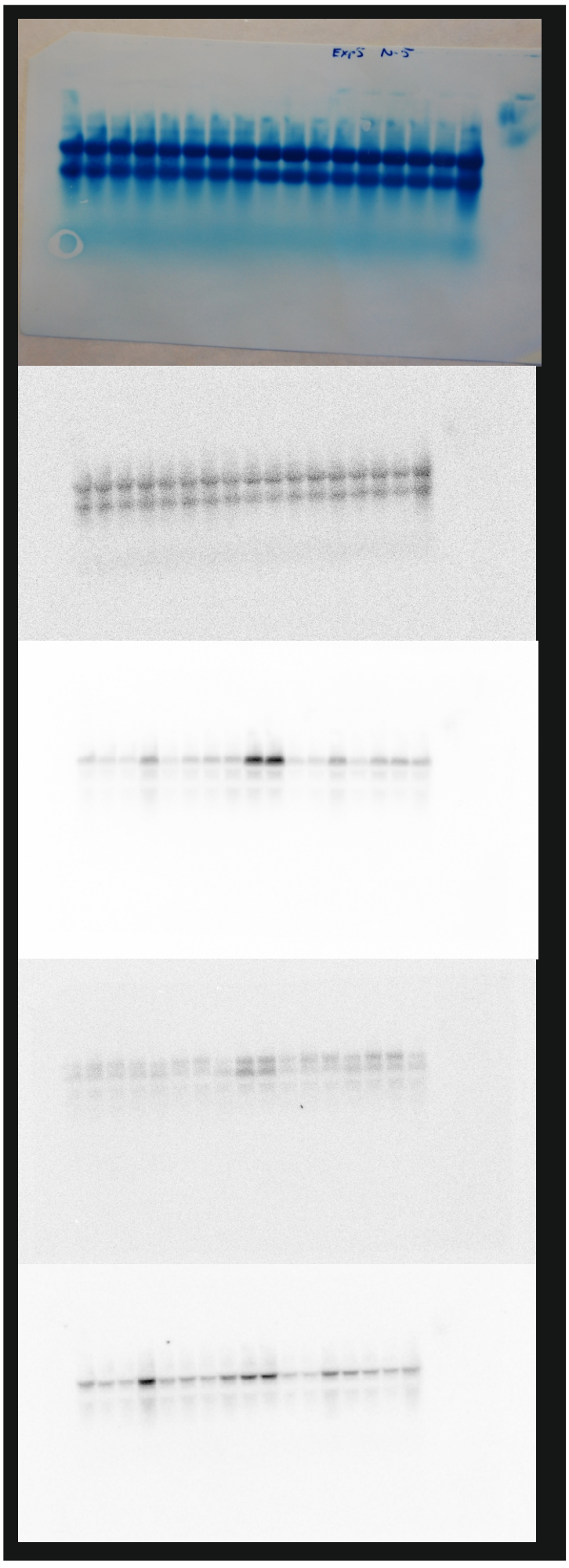
probe *enaA*

probe *enaB*

probe *enaC*

probe *msnA*

Full size images for hybridations of *enaA*Δ samples filter
See Figure 3 panel C and legend for lanes information.



rRNA

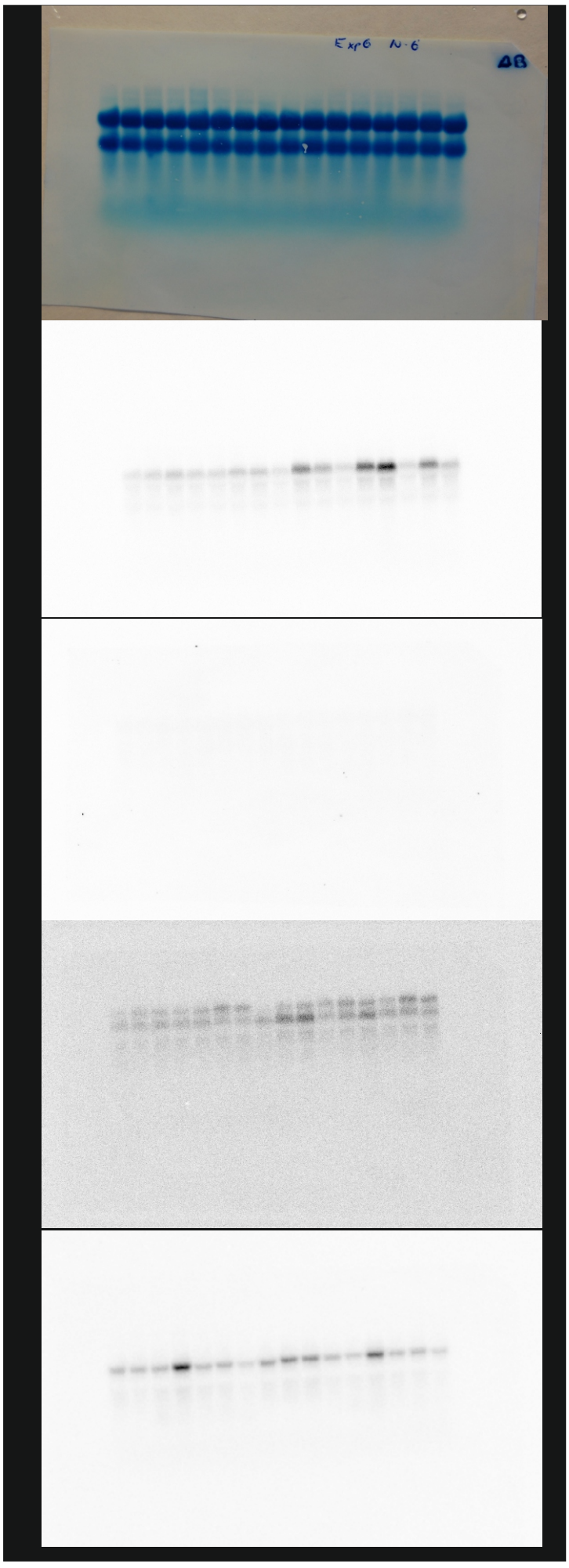
probe *enaA*

probe *enaB*

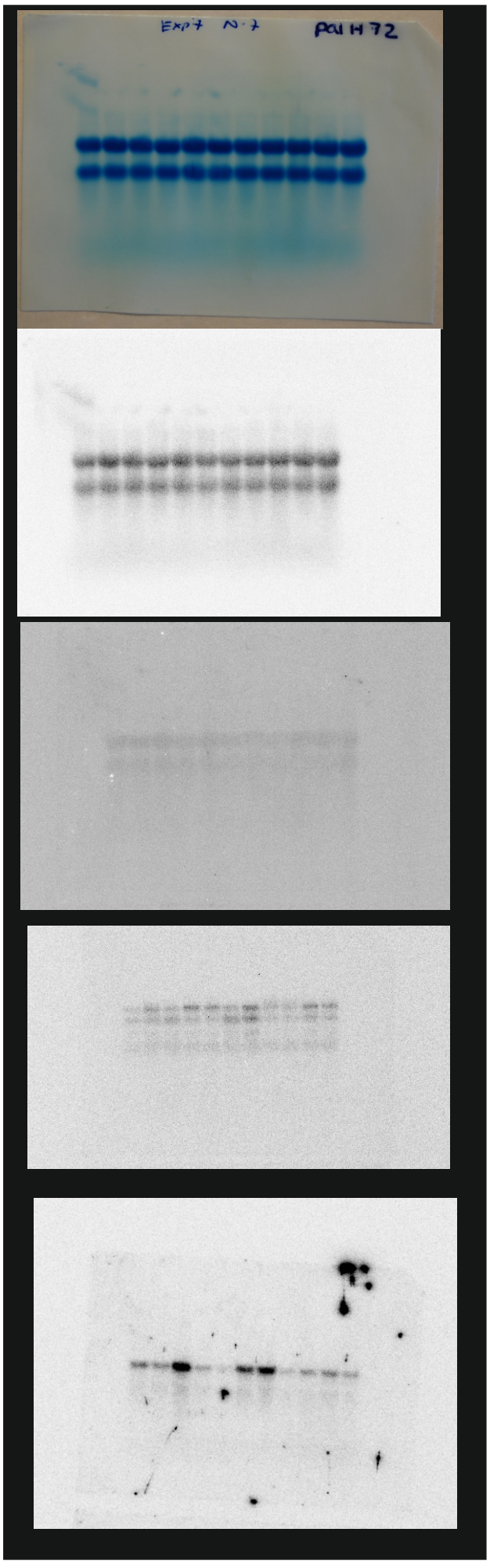
probe *enaC*

probe *msnA*

Full size images for hybridations of *enaB*Δ samples filter
See Figure 3 panel D and legend for lanes information.



Full size images for hybridations of *palH72* samples filter
See Figure 4 panel B and legend for lanes information.



rRNA

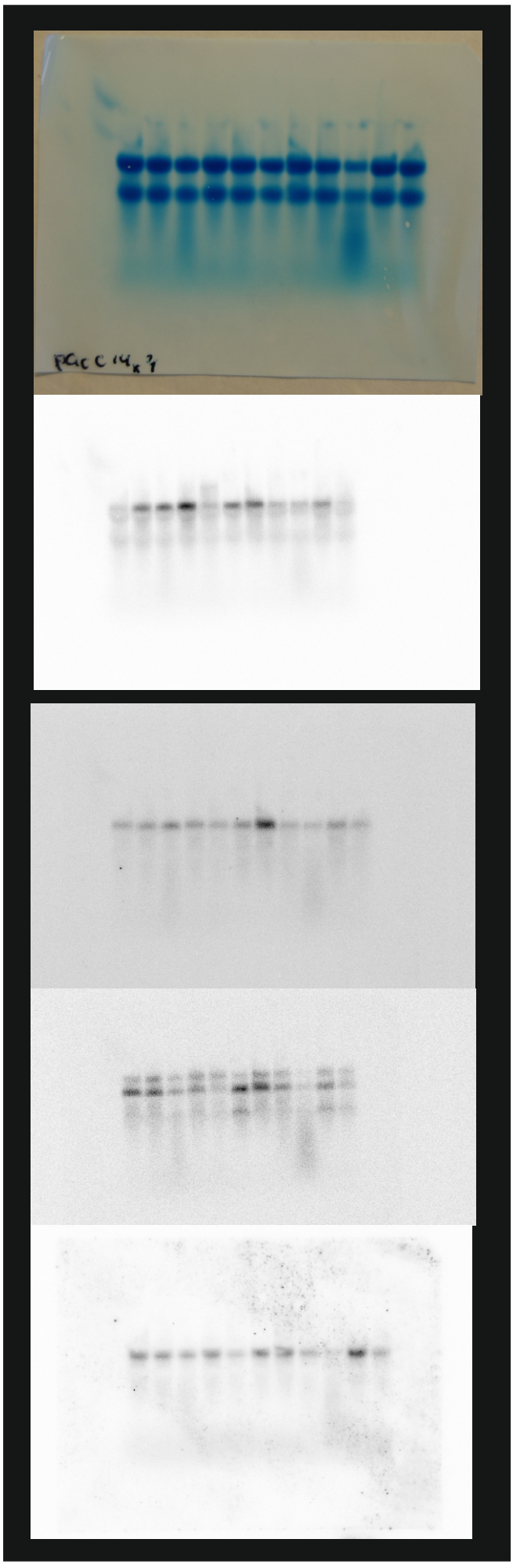
probe *enaA*

probe *enaB*

probe *enaC*

probe *msnA*

Full size images for hybridations of *pacC*^{c}*14* samples filter
See Figure 4 panel C and legend for lanes information.



rRNA

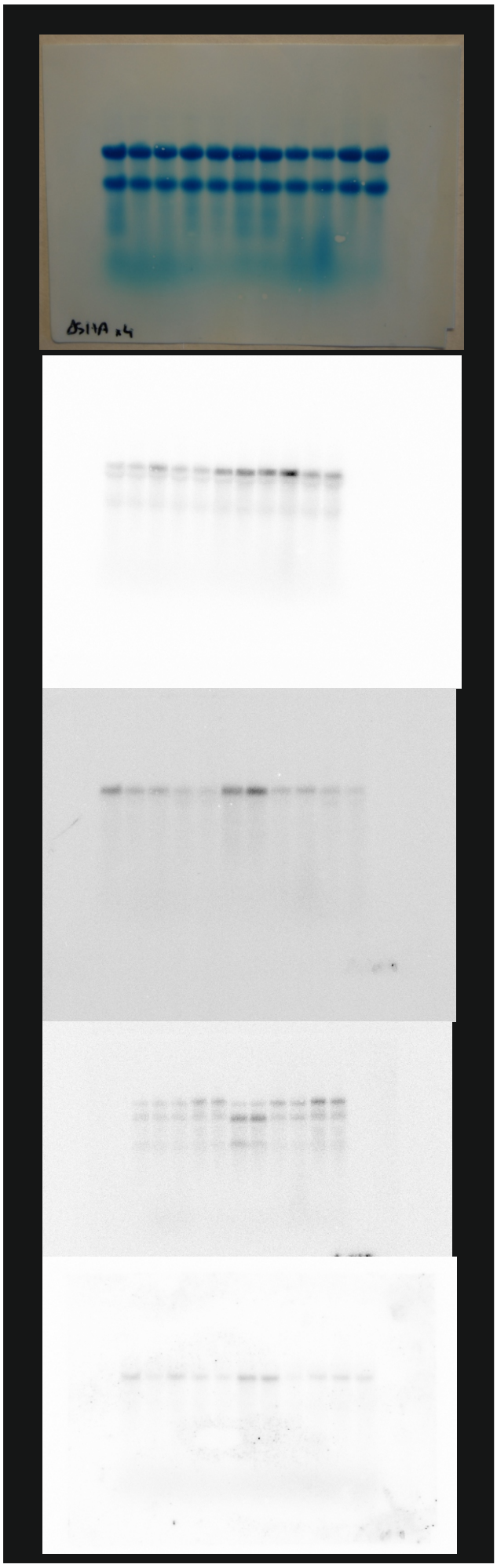
probe *enaA*

probe *enaB*

probe *enaC*

probe *msnA*

Full size images for hybridations of *sltA*Δ samples filter
See Figure 4 panel D and legend for lanes information.



rRNA

probe *enaA*

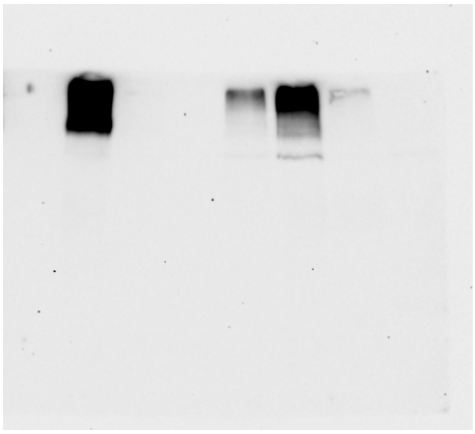
probe *enaB*

probe *enaC*

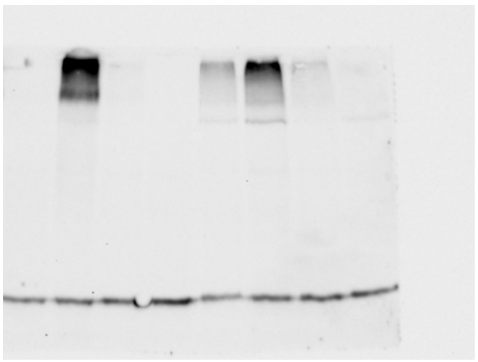
probe *msnA*

Full size images for MW markers on filter and immunodetection of GFP and actin in protein extracts from EnaA and EnaB-GFP expressing strains. See Figure 5 panel A and legend for lanes information.

Filter with prestained MWs

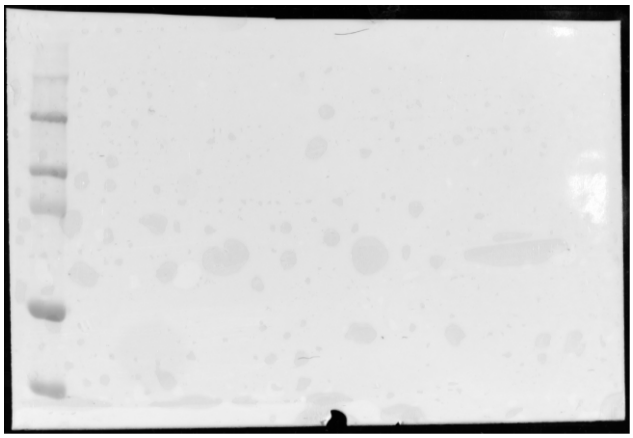


anti-GFP

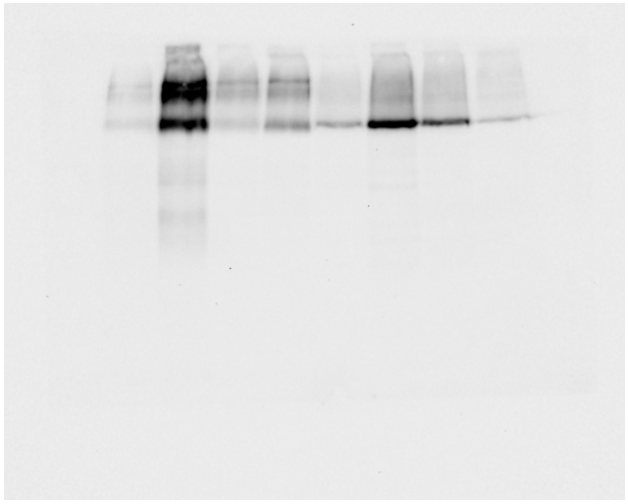


antiGFP + anti-actin

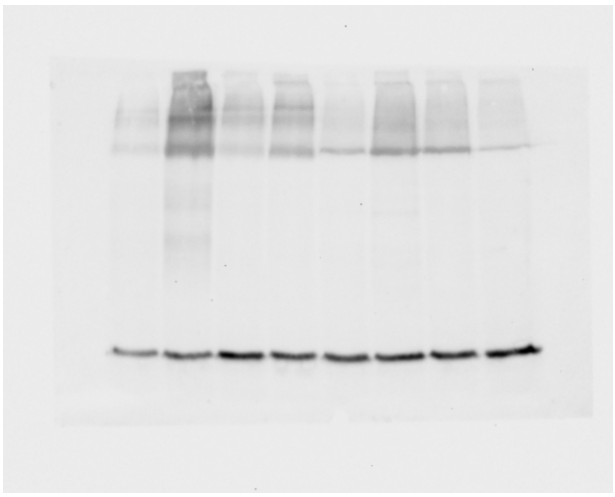
Full size images for MW markers on filter and immunodetection of GFP and actin in protein extracts from EnaA and EnaB-GFP expressing strains. See Figure 5 panel B and legend for lanes information.



Filter with prestained MWs

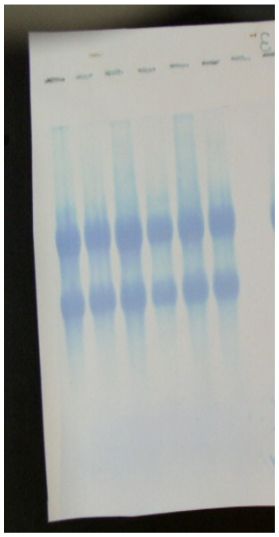


anti-GFP



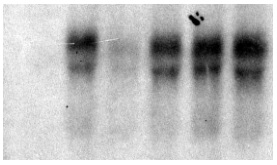
antiGFP + anti-actin

Full size images for hybridations of *gpdA* driven *enaA-gfp* chimera
See Figure 7 panels A and B, and legend for lanes information.



rRNA

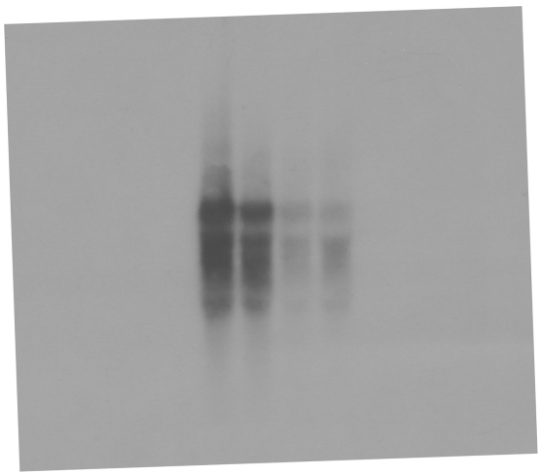
Panel
WT background



probe *enaA*

Panel B,
palA1 background

rRNA



probe *enaA*