Developmental Cell *14*

Supplemental Data

Ajuba LIM Proteins Are Snail/Slug Corepressors Required for Neural Crest Development in *Xenopus*

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Supplemental Experimental Procedures

Generation of P19-siRNA Cell Lines

Retroviral shRNA constructs targeting Luciferase (control) or mouse Ajuba (nucleotides 702-720) were generated by PCR in the pSUPERretro vector (Oligoengine). For lentiviral production, HEK293T cells were cotransfected with retroviral construct and packaging vectors (pHR'8.2deltaR/pCMV-VSV-G at ratio 8:1). P19 cells were infected using standard protocols and selected in puromycin.

Plasmids

pCS2.myc-mAjuba, -mLimd1, -LPP, -Zyxin, -mAjubaPreLIM, -mAjubaLIM, pcDNA3.1.RFP-mAjuba, and have been described previously (Goyal et al. 1999; Marie et al. 2003; Pratt et al. 2005; Feng et al. 2007). Flag-tagged mLIMD1 mutant constructs were subcloned into pcDNA3.1. pCMV14.mSnail-Flag was a kind gift from H. L. Grimes (University of Louisville). pCS2.myc-mWTIP as well as pCMV5.HA-mSnail full-length and ΔSNAG constructs and pcDNA3.1.YFP-hSnail.ΔSNAG were generated through PCR. GFP-hSnailWT and GFP-hSnail8SA were subcloned into pcDNA3.1 (Invitrogen) from the corresponding CMV-Tag constructs, which were from M. Hung (University of Texas M.D. Anderson Cancer Center). pCS2-XSlug, -XSlZnF, and –

EngRXSlZnF were from C. LaBonne (Northwestern University) and XSnail was isolated by PCR from an X. laevis cDNA library, and subcloned into pCS2-myc. All cloning was confirmed by DNA sequencing.

Cloning of the Xenopus Ajuba LIM Protein Orthologs

An EST database search revealed partial sequences of Zyxin (BU907427, BG234393, BJ068010, BI349606, and BG409548), LPP (BC077865, BU903395, CB983325, BG363738, BU912724, BM179756, and BG364767), LIMD1 (BU914068 and AW642119), and WTIP (DY560028, BJ042412, BJ068924 and AW645564). Phylogenetic relationships between the LIM regions of M. musculus, D. melanogaster, C. elegans, and putative X. laevis LIM proteins were generated using the Clustal W algorithm and MegAlign software (DNASTAR). To clone *X. laevis* orthologs of Ajuba family members, gene-specific primers to full length XLIMD1 and XWTIP were designed based on available EST sequences. Full-length XLIMD1 and XWTIP cDNAs were amplified from a *X. laevis* stage 24 embryo cDNA library by PCR. These fragments were cloned into pCS2 and sequenced (XLIMD1 Gen Bank Accession DQ913740, XWTIP Gen Bank Accession EU257484).

Primer Sequences for RT-PCR and ChIP

For RT-PCR in P19 cells, primers were as follows: E-cadherin sense: 5'-GAGAACGGTGGTCAAAGAGC-3'; antisense: 5'-CATCTCCCATGGTGCCACAC-3'. GAPDH sense: 5'-ACCACAGTCCATGCCATCAC3'; antisense : 5'-TCCACCCCCTGTTGCTGTA-3'.

For ChIP in human HEK293T cells, immunoprecipitated DNA was amplified using Primer set 1 (sense: 5'-AATCAGAACCGTGCAGGTCC-3'; antisense: 5'-

ACAGGTGCTTTGCAGTTCCG-3') to amplify the E-cadherin promoter or Primer set 2 (sense: 5'-GGCTCAAGCTATCCTTGCAC-3'; antisense: 5'-GTGCAGTGGCTCAT-GTCTGT-3') to amplify a control region in Exon 16.

For ChIP in mouse P19 cells, immunoprecipitated DNA was amplified using Primer set 1 (sense: 5'- TAG GCT AGG ATT CGA ACG ACC G-3'; antisense: 5'-TCT TGG GAA CTC AGT AGT GCG C-3') to amplify the E-cadherin promoter or Primer set 2 (sense: 5'-AGGTATCTTGGTGTGGGTGCAACT-3'; antisense: 5'-ACGCCAAGAAACTTAAGTGGTGCC-3') to amplify a control region in Exon 15.

In Situ Hybridization

Sense and antisense *in situ* probes of each XLIMD1 or XWTIP clone and antisense probes of Sox2 (Mizuseki et al. 1998), Epidermal keratin (Epiker) (Jonas et al. 1985), Slug (Mayor et al. 1995), Twist (Hopwood et al. 1989), Snail (Essex et al. 1993), Pax3 (Bang et al. 1997), c-myc (Bellmeyer et al. 2003), and FoxD3 (Bellmeyer et al. 2003) were generated by *in vitro* transcription with digoxigenin-11-UTP (Roche). For XLIMD1 *in situs*, two IMAGE clones of XLIMD1, 5513507 and 6640526, that overlap the preLIM region (nts -97-677) and LIM region (nts 1330-1836) were used. For XWTIP in situs, IMAGE clone 8329059 that overlaps the preLIM region (nts -196-554) was used.

For double *in situs*, Dig-labeled XLIMD1 probe (6640526) was used in combination with a probe to Slug generated by *in vitro* transcription with fluorescein-12-UTP (Roche). Probes were detected using AP-anti-digoxigenin (Roche) with BCIP (Roche) and AP-anti-fluorescein (Roche) with Magenta phosphate (Biosynth).

XLIMD1 and XWTIP Morpholinos

The XLIMD1 MO (5'-ACTGTTTAACGGCAAAATAATTCTG-3') recognizes a region in 5'UTR (45–69 nucleotides upstream from start), and the XWTIP MO (5'-TTCTC**CAT**GTCCTGCCTCCTCTTCC-3') recognizes a region that crosses the start site (in bold).

Supplemental References

Bang, A.G., Papalopulu, N., Kintner, C., and Goulding, M.D. 1997. Expression of Pax-3 is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non-axial mesoderm. *Development* **124**(10): 2075-2085.

Bellmeyer, A., Krase, J., Lindgren, J., and LaBonne, C. 2003. The protooncogene c-myc is an essential regulator of neural crest formation in xenopus. *Dev Cell* **4**(6): 827-839.

Essex, L.J., Mayor, R., and Sargent, M.G. 1993. Expression of Xenopus snail in mesoderm and prospective neural fold ectoderm. *Dev Dyn* **198**(2): 108-122.

Feng, Y., Zhao, H., Luderer, H.F., Epple, H., Faccio, R., Ross, F.P., Teitelbaum, S.L., and Longmore, G.D. 2007. The LIM protein, Limd1, regulates AP-1 activation through an interaction with Traf6 to influence osteoclast development. *J Biol Chem* **282**(1): 39-48.

Goyal, R.K., Lin, P., Kanungo, J., Payne, A.S., Muslin, A.J., and Longmore, G.D. 1999. Ajuba, a novel LIM protein, interacts with Grb2, augments mitogen-activated protein kinase activity in fibroblasts, and promotes meiotic maturation of Xenopus oocytes in a Grb2- and Ras-dependent manner. *Mol Cell Biol* **19**(6): 4379-4389.

Hopwood, N.D., Pluck, A., and Gurdon, J.B. 1989. A Xenopus mRNA related to Drosophila twist is expressed in response to induction in the mesoderm and the neural crest. *Cell* **59**(5): 893-903.

Jonas, E., Sargent, T.D., and Dawid, I.B. 1985. Epidermal keratin gene expressed in embryos of Xenopus laevis. *Proc Natl Acad Sci U S A* **82**(16): 5413-5417.

Marie, H., Pratt, S.J., Betson, M., Epple, H., Kittler, J.T., Meek, L., Moss, S.J., Troyanovsky, S., Attwell, D., Longmore, G.D., and Braga, V.M. 2003. The LIM protein Ajuba is recruited to cadherin-dependent cell junctions through an association with alpha-catenin. *J Biol Chem* **278**(2): 1220-1228.

Mayor, R., Morgan, R., and Sargent, M.G. 1995. Induction of the prospective neural crest of Xenopus. *Development* **121**(3): 767-777.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S., and Sasai, Y. 1998. Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**(4): 579-587.

Pratt, S.J., Epple, H., Ward, M., Feng, Y., Braga, V.M., and Longmore, G.D. 2005. The LIM protein Ajuba influences p130Cas localization and Rac1 activity during cell migration. *J Cell Biol* **168**(5): 813-824.

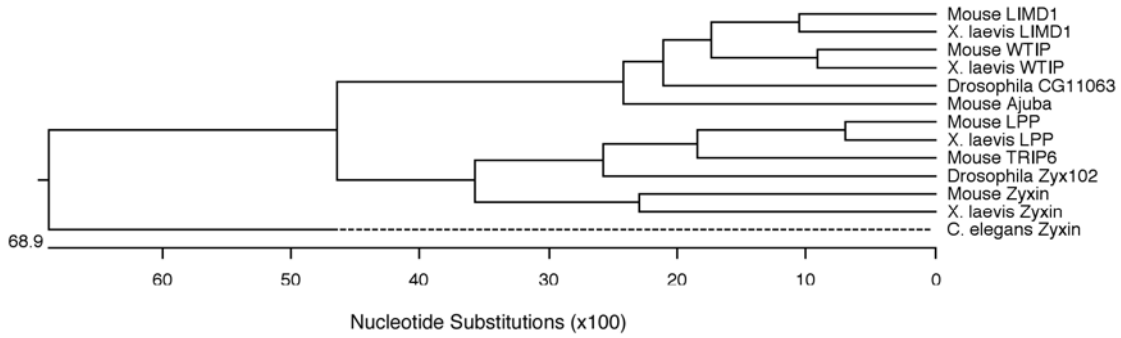

Figure S1. *X. laevis* Orthologs of the Ajuba and Zyxin Families of LIM Proteins

A predicted phylogenetic relationship of the LIM regions of the C. elegans, Drosophila, mouse and putative X. laevis orthologs of the Ajuba/Zyxin family of LIM proteins. Phylogenetic relationships between the LIM regions were generated using the Clustal W algorithm and MegAlign software (DNASTAR).

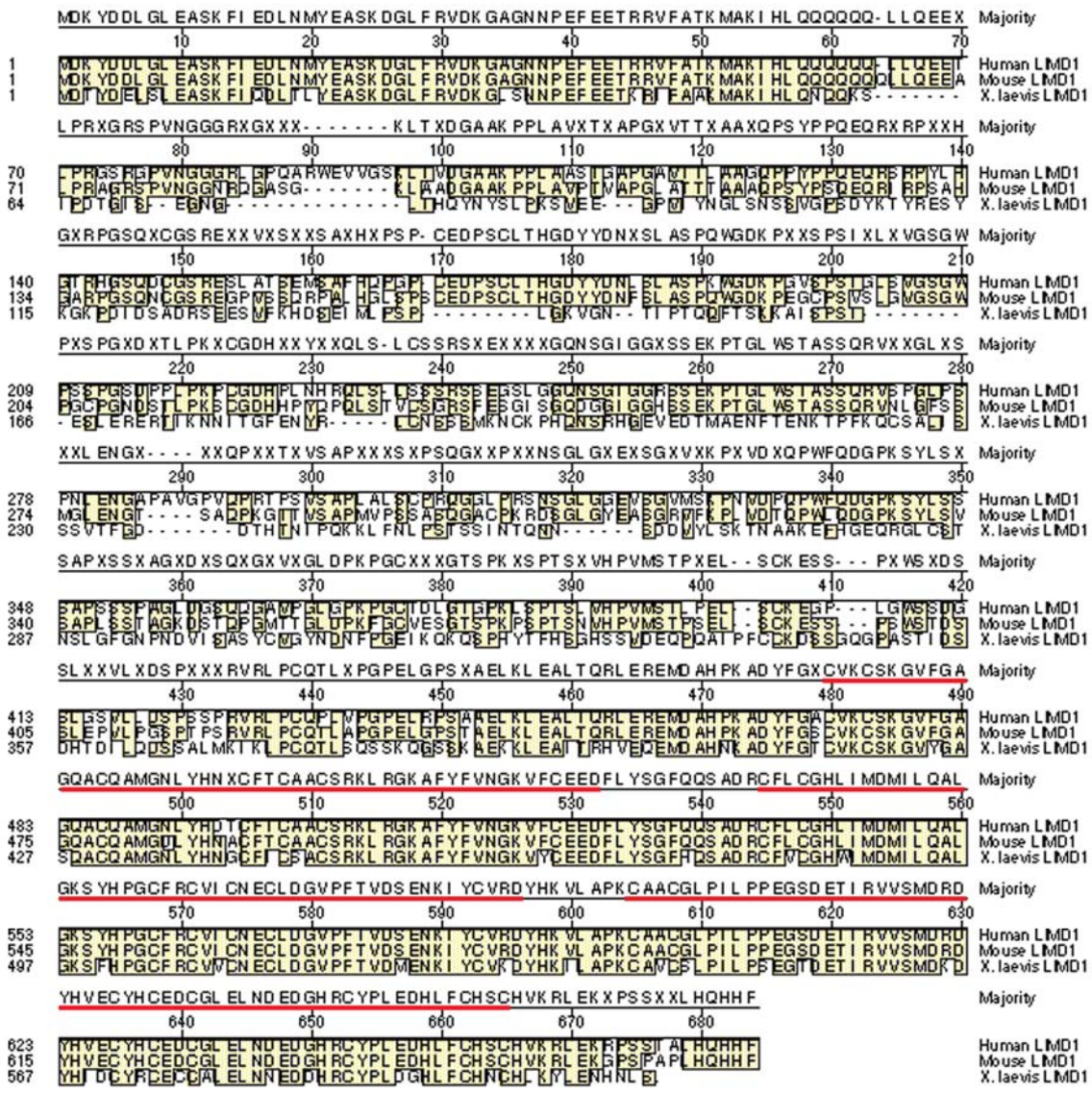

Figure S2. Alignment of Full-Length *X. laevis*, Mouse, and Human LIMD1 Sequences

Consensus sequence is boxed in yellow where it exists. The three C-terminal LIM domains are underlined in red. In the LIM regions, XLIMD1 has 83% identity to mLIMD1 at the amino acid level.

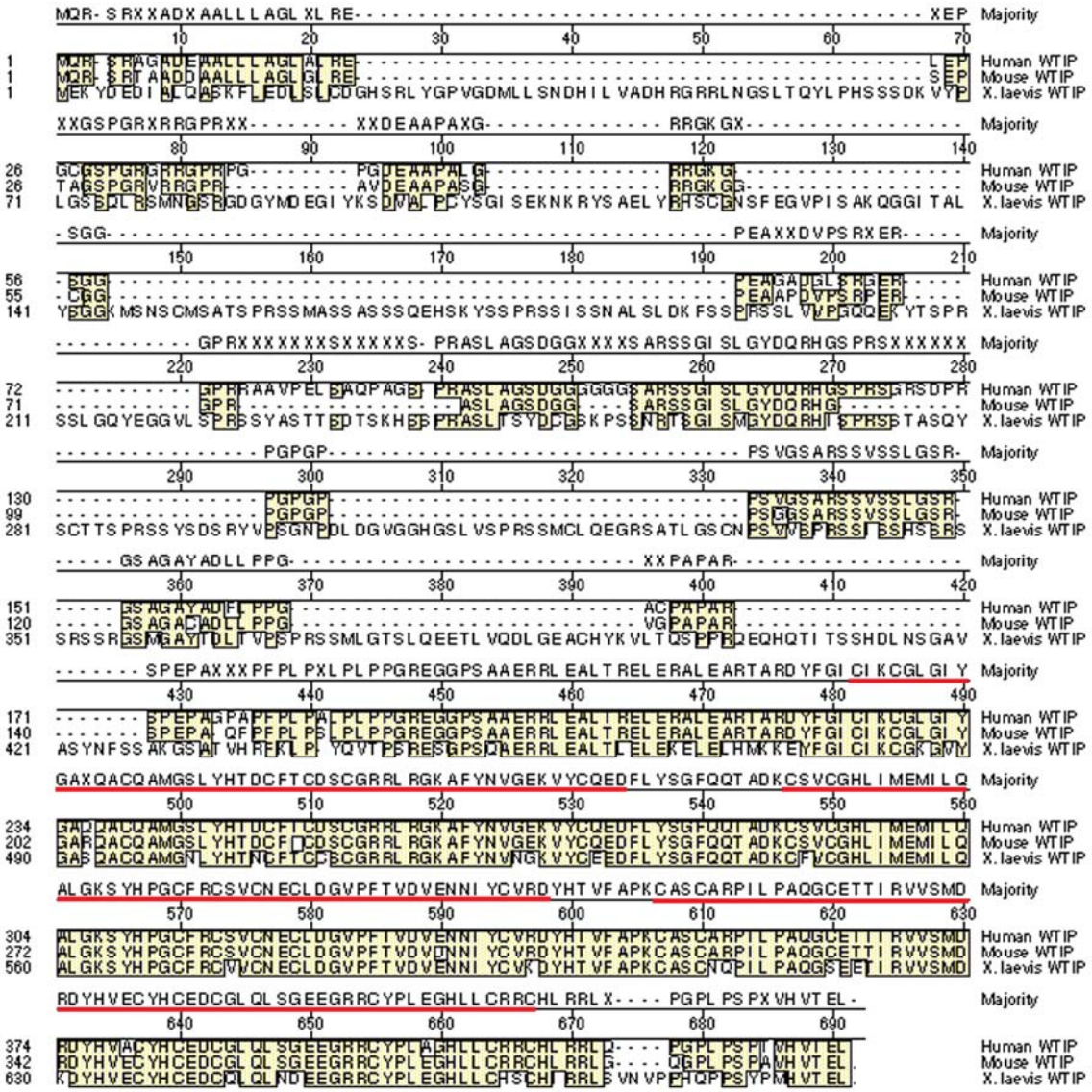

Figure S3. Alignment of Full-Length *X. laevis*, Mouse, and Human WTIP Sequences

Consensus sequence is boxed in yellow where it exists. The three C-terminal LIM domains are underlined in red. In the LIM regions, XWTIP has 82% identity to mWTIP at the amino acid level.

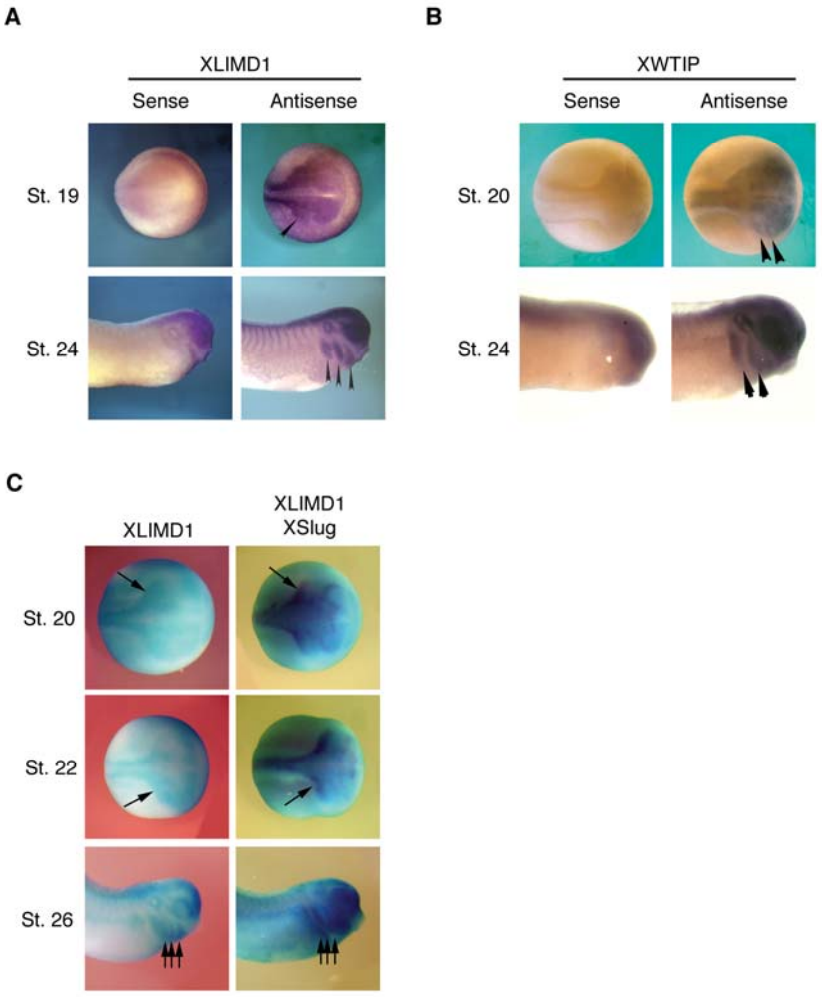

Figure S4. Endogenous XLIMD1 and XWTIP Expression Patterns Overlap that of XSlug

A,B. *In situ* hybridization was performed on uninjected X. laevis embryos using sense and antisense probes for XLIMD1 (A) or XWTIP (B) at stages shown. Low level, non-specific staining of XLIMD1 and XWTIP was seen in blastula and gastrula stage embryos and by stage 13.5, these proteins were enriched throughout the neural plate (not

shown). In stage 19/20 embryos (top panels), XLIMD1 and XWTIP were enriched in the territory encompassing the anterior neural plate and the premigratory neural crest. By tailbud stages (lower panels), there was strong expression of XLIMD1 and XWTIP in the migrating neural crest, as well as in the eye, brain, and otic placode. Arrowheads indicate staining in the premigratory and migratory neural crest. Embryos are oriented with dorsal up and anterior to the right. C. Double *in situ* hybridization for XLIMD1 and XSlug in X. laevis embryos at stages shown. XLIMD1 localization alone (blue) is shown on the left and XSlug is overlayed (pink) on the right. Arrows indicate areas of colocalization (purple) in neural crest derivatives. Embryos are oriented with dorsal up and anterior to the right.

Table S1. Ajuba LIM Proteins Affect *Xenopus* Neural Crest Early in Its Development

	Pax3 (St12)	Snail (St12)	c-myc (St16)	FoxD3 (St16)	Slug (St18)	Twist (St18)
Control MO	0	7	0	0	3	3
XLIMD1 MO (10ng)	44	56	24	44	31	57
XWTIP MO (5ng)	85	70	58	92	67	68
XSlug MO (10ng)	76	47	59	68	ND	78

Table showing the percent of embryos with decreased neural crest on the injected side following injection of Control, XLIMD1, XWTIP, or XSlug MOs as shown. Neural crest development was assayed through *in situ* hybridization for neural crest markers Pax3, Snail, c-myc, FoxD3, Slug, and Twist at stages indicated.