

Genomic Signatures Predict Migration and Spawning Failure in Wild Canadian Salmon

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Long-term population viability of Fraser River sockeye salmon (*Oncorhynchus nerka*) is threatened by unusually high levels of mortality as they swim to their spawning areas before they spawn. Functional genomic studies on biopsied gill tissue from tagged wild adults that were tracked through ocean and river environments revealed physiological profiles predictive of successful migration and spawning. We identified a common genomic profile that was correlated with survival in each study. In ocean-tagged fish, a mortality-related genomic signature was associated with a 13.5-fold greater chance of dying en route. In river-tagged fish, the same genomic signature was associated with a 50% increase in mortality before reaching the spawning grounds in one of three stocks tested. At the spawning grounds, the same signature was associated with 3.7-fold greater odds of dying without spawning. Functional analysis raises the possibility that the mortality-related signature reflects a viral infection.

For 60 years preceding the early 1990s, approximately 8 million sockeye salmon (*Oncorhynchus nerka*) returned annually from the Pacific Ocean to Canada's Fraser River basin to spawn. However, since then, sockeye salmon productivity has declined precipitously to the point that returns in 2009 were less than the replacement rate. Consequently, the long-term viability of the wild salmon resource in British Columbia, worth over \$1 billion dollars annually, is in doubt. Indeed, several of these Canadian stocks are at risk of extinction (1, 2). In 2009, the prime minister of Canada announced a judicial inquiry into this salmon collapse, which has occurred despite substantial reductions in fisheries harvest. Contributing to the collapse have been massive (40 to 95%) mortalities of adult sockeye salmon before spawning, both in the Fraser River en route to spawning areas and on spawning grounds (3). The causal mechanisms of this premature mortality have eluded multidisciplinary research by scientists and fisheries managers (4). However, the three functional

genomics studies presented here reveal a striking and consistent association between a powerful genomic signature and salmon mortality.

Seven of the last 10 summers have been the warmest on record for the Fraser River, and

biotelemetry has revealed high losses of migrating sockeye in regions of elevated river temperature (5). Warmer water reduces the delivery of oxygen to the tissues (aerobic scope) of salmon (6) and allows more rapid development of infections (7). Our preliminary studies also suggest that some fish are stressed before they reach the river, further impairing their survival (8). The current study was undertaken to advance our mechanistic understanding of the role of salmon condition (before mortality events occur) on migration and spawning success in the river. We combined established methodologies of nonlethal biopsy of ocean- and river-caught salmon with watershed-scale biotelemetry to follow the fate of tagged fish migrating upstream (9, 10). Functional genomics and tracking of individuals were used to correlate physiological profiles with failed migrations and reproduction. Gene expression was profiled in gill tissue, a respiratory and ionoregulatory organ that is highly responsive to stress, chemical exposure, and disease.

Returning adult salmon caught in the ocean and river were gastrically implanted with a radio transmitter—or a Peterson disc tag if caught at spawning areas—and biopsied for blood, gill, muscle, and fin tissues (10); fin tissue was used to genetically identify sockeye stocks (11). We tracked individual fish with radio-receivers deployed

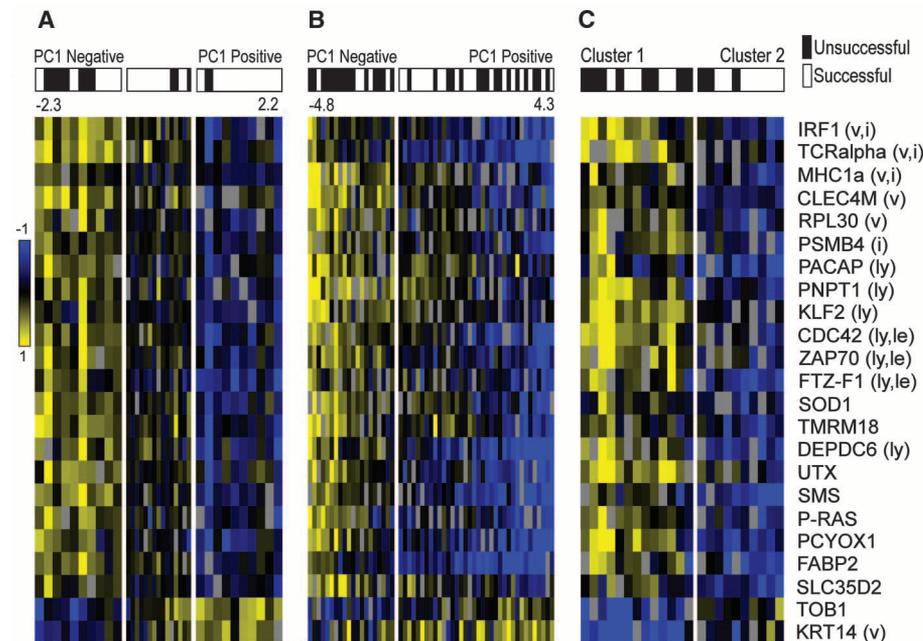


Fig. 1. Heatmaps of 23 annotated genes significantly associated with survivorship in all three studies. For (A) ocean-tagging and (B) freshwater-tagging studies, heatmaps reflect the ranking of individuals along the PC1 axis (rotational values shown above the heatmap), in which the associations with fate were strongest at the ends of the PC1 distribution, which are demarcated by white blocks. Migration success, depicted in the top bar, was reduced at the PC1-negative end of the axis for both studies. (C) For the spawning study, the heatmap reflects the relationships depicted by clustering significant genes from the freshwater PC1-based *t* test, with the white block differentiating the two emergent clusters and the black/white bar reflecting unsuccessful and successful spawners, respectively. (Right) Literature associations of genes with viruses (v), immune response (i), lymphocytes (ly), and leukemia (le) are depicted with letter codes in parentheses next to gene names. (Left) Expression levels are indicated by the color scale ranging from (up-regulated) yellow to (down-regulated) blue. Missing values are shown in light gray.

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throughout the Fraser watershed (fig. S1) to identify date of river entry (for ocean-tagged fish) and in-river fate (location the fish was last detected). Expression profiles were compared between fish that arrived at spawning areas (successful migrants) with those that perished en route. Previous biotelemetry data showed that large losses of sockeye in the upper river (above Hells Gate) (fig. S1) cannot be attributed to river fisheries, which are largely restricted to the lower river (12). Thus, to minimize interference from fisheries activities we contrasted expression profiles

only for survivors and fish disappearing above Hells Gate in the ocean-tagging study ($n = 35$ salmon), comprising Late Shuswap Adams fish released 215 and 300 km from the river mouth, in Johnstone Strait and Juan de Fuca Strait, respectively (fig. S1 and table S1). The larger freshwater-tagging study ($n = 104$ salmon) occurred 69 km upstream of the river mouth on Late Shuswap (largely Adams), Chilko, and Scotch Creek stocks that perished throughout the Fraser River drainage but survived at least 2 days after tagging (to minimize tagging and handling effects). Because large

numbers of fish [for example, >80% (3)] can die on the spawning areas before spawning, we tagged fish at the Weaver Creek spawning area (fig. S1) and compared the genomic signatures of 11 failed and 12 successful spawners. The ocean and freshwater studies used a salmonid 16K feature cDNA microarray (13, 14), in which 11,535 of the 16,008 genes have gene annotations, whereas the spawning study used a salmonid 32K feature cDNA microarray (15), which contained an additional 16K genes, 7513 with gene annotations (16, 17).

Fig. 2. Survivorship analysis revealed a significant interaction between stock and PC1 in fresh water. **(A)** Graphical representation of PC1 to PC10. **(B to D)** Survivorship curves for Scotch Creek, Chilko, and Late Shuswap Adams, respectively. Although in the analysis the value of PC1 was taken as a continuous variable, to graphically represent the correlation with survival the PC1 rotational values were divided into negative (<0, black line) and positive (>0, red line) categories.

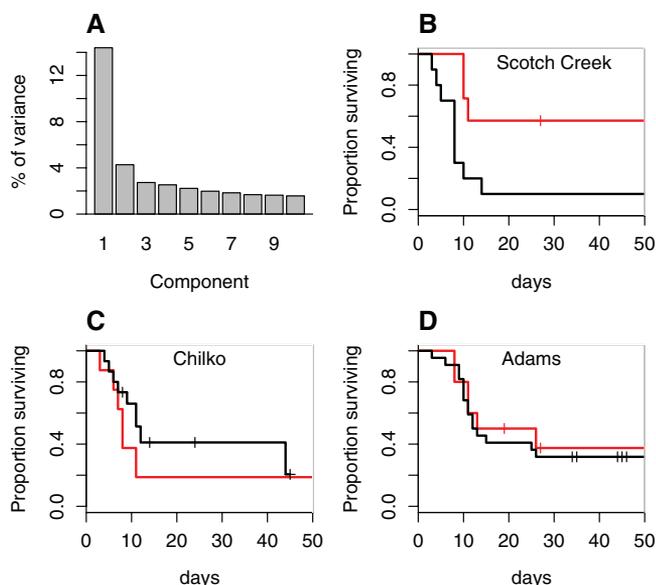
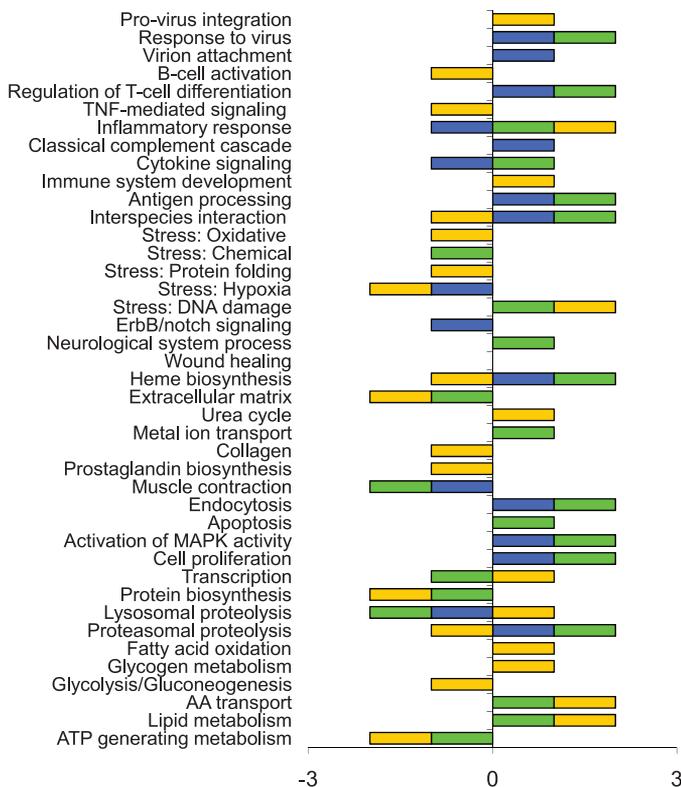


Fig. 3. Functional analysis of signatures associated with fate in (blue) saltwater, (green) freshwater, and (yellow) spawning studies. The axis indicates the number of studies in which a biological process (defined as a collection of molecular events with a defined beginning and end) was (positive) up- or (negative) down-regulated in the mortality- versus survivor-related PC1 signature. Biological processes differentially regulated with no clear indication of overall direction are not shown. A more detailed presentation is available in table S3.



Supervised analyses of the ocean-tagging data (analysis of variance and computer algorithm) to detect genes differentially expressed between successful and unsuccessful migrants did not yield a significant result, suggesting that a single physiological mechanism was not likely to be responsible for all river mortality. Alternately, by taking an unsupervised principal component (PC) analysis approach we identified the underlying gene expression patterns in the data and assessed the top five PCs for associations with fate. Among the top five PCs, only PC1 (explaining 12% of the variance in the data) yielded a ranking of fish that showed a significant correlation with survival (Mann-Whitney $U = 183$, with $P = 0.03$). Further data inspection revealed a complex relationship between fate and PC1, with enrichment at the negative and positive ends of the PC1 distribution, encompassing approximately 60% of the fish in the study (Fig. 1A). Upper river mortalities were twice as common in the PC1 negative and three times less common in the PC1 positive ends, corresponding to an odds ratio (OR) of 13.5. Moreover, arrival at the receiver adjacent to Adams River spawning areas was on average 15 days faster for successful PC1-negative migrants than successful PC1-positive migrants, 10 days faster after river entry. In 2006, successful spawners also swam upstream slower than fish that failed [20.0 versus 15.5 km/day (18)]. Taken together, these results showed that up to 60% of fish contained a gene expression signature in seawater >200 km from the river that was predictive of in-river fate, which in 2006 represented over 2.4 million Late Shuswap fish.

We hypothesized that a similar pattern would exist in the freshwater-tagging study. Comparing only successful migrants and upper-river mortalities ($n = 56$ salmon), the first PC of the freshwater-tagging data was related to PC1 of the ocean-tagging data (see below). Again, an over-representation of unsuccessful migrants was apparent on the extreme PC1-negative end of the distribution, with the odds of successful migration five times lower in the first third of PC1-negative fish as compared with all remaining fish in the study (χ^2 , $P < 0.05$) (Fig. 1B). PC2 to PC5 showed no correlation with survival, and supervised analyses did not yield a significant result (17).

The larger freshwater-tagging study included fish that went missing throughout the Fraser River and sufficient sample sizes from three salmon stocks so as to facilitate a more precise analytical

approach. Using all but known fisheries losses for these stocks [$n = 72$ salmon; see (17) for fisheries analyses], the first four PCs along with stock and sex were included as explanatory variables in survivorship analysis (17). Parametric survival analysis revealed a significant stock *PC1 interaction [$F_{2,65} = 7.30$, $P = 0.026$]. Further analysis revealed a significant relationship between PC1 and survival for Scotch Creek fish [$F_{1,14} = 4.97$, $P = 0.026$] but not for Chilko or Late Shuswap salmon ($P > 0.05$) (Fig. 2). Although the stock *PC3 interaction [$F_{2,65} = 6.44$, $P = 0.040$] was also significant, the relationship was not significant when individual stocks were considered (17). PC2, PC4, and sex were not explanatory. Differences observed among stocks suggest that some are more severely affected than others. However, other influences could include stock-specific differences in travel time to the receiver adjacent to spawning tributaries (averaging 12, 17, and 24 days, respectively, for Chilko, Scotch, and Late Shuswap), travel time from last receiver to spawning areas (7 days Chilko versus 1 day Scotch/Late Shuswap), and levels of subsequent mortality on spawning areas.

To obtain groups of significant genes for functional analysis, we used t tests to compare samples in the extreme PC1-positive and -negative quartiles for both studies. A reproducible pattern of gene expression correlated with fate emerged. 1603 genes were significant at $P < 0.001$ in saltwater, and 2762 genes were significant in fresh water. 498 genes were common to both independent data sets, of which 97% were directionally congruent and 90% were up-regulated in PC1-negative fish (fig. S2).

To test the hypothesis that the same genomic signature was also associated with premature mortality at the Weaver Creek spawning area, we used the significant genes from the freshwater t test to cluster successful and unsuccessful spawners. Two well-differentiated clusters emerged, with >70% of unsuccessful spawners in the cluster associated with the PC1-negative mortality-related signature. Salmon with this signature were 3.7 times less likely to spawn than those with a PC1-positive-related signature, despite reaching the spawning area. To assess changes in the signature that may occur closer to spawning, we conducted a t test between the two clusters; 2507 significant genes were resolved ($P < 0.001$), of which 1136 were on the 16K array, with 36% overlap for freshwater and/or saltwater t test gene lists, 98% of which were directionally congruent (Fig. 1C and fig. S2). The correlation between genomic signatures associated with poor survival throughout return migration was further supported by quantitative reverse transcription polymerase chain reaction validation of six genes (APR-3, ATP6V1C1, FKBP2, C4B, SCHC, and CYP46A1) that showed even more consistent up-regulation in the mortality-related signature fish in all three tagging studies than revealed on microarrays (table S2).

Physiological differentiation along the PC1 axis escalated appreciably during migration into

the river and toward spawning areas, with 25 biological processes differentially affected in the ocean, 34 in fresh water, and 47 at spawning (Fig. 3 and table S3) (17). Furthermore, an intensification of complement-mediated inflammatory and perforin-mediated apoptotic processes occurred for fish containing the mortality-related signature. Immune stimulation of these same fish was indicated by T-cell activation/proliferation and induction of a Th1 cellular immune response through interferon activation of the virus-specific innate JAK/STAT pathway. Some of the most consistent and/or significantly up-regulated genes (such as Mx, STAT1, IRF1, PRF1, MHC1a, PCSK5, and TCR α) have known linkages with viral activity (Fig. 1C and table S4). Moreover, 65% of affected biological processes were consistent with responses to viral infections (table S3); within these processes, many key regulators co-opted by or activated in response to viruses were differentially expressed (17). These data indicate that fish containing the genomic signature correlated with elevated mortality may be responding to viral infection [details are in (17)]. Linkages also existed with genes associated with leukemia, most notably cell lymphoblastic leukemia-lymphoma (fig. S3 and tables S3 and S4).

This correlative data set cannot be used to assign cause to the association between a preexisting signature and subsequent mortality. However, we can eliminate the possibility that this signature simply relates to the inevitable senescence of salmon after spawning because the mortality-survival-associated PC1 signature showed relatively stronger differentiation on the spawning area, when salmon were within 1 to 3 weeks of death, than in the ocean, when salmon were 3 to 10 weeks from death. The relatively stronger association with survival in the ocean-tagging study also suggests tagging effects did not appreciably influence the genomic relationships of PC1 with mortality (17). Moreover, because the mortality-related signature preexisted before river entry, it cannot reflect a response to stress of moving from seawater to fresh water. In fact, few indications of a general stress response existed within the mortality-related signature; DNA damage was the only stress-specific biological process up-regulated, as indicated by elevated expression of more than 20 genes (such as KIN, RAD51, CRY5, and NSMCE2) (Fig. 3). However, these fish could have experienced salinity stress in seawater induced by a premature transcriptional shift in osmoregulatory genes [Na⁺/K⁺ adenosine triphosphatase (ATPase) isoforms 1a, 1b, and a3 (fig. S5) and PRL, SHOP21, CIRBP, CLIC5 SLC5A1, and FXVD3] better suited for fresh water (17). Indeed, elevated chloride and osmolarity were anti-correlated with PC1 of ocean-tagged fish (Spearman rank = -0.33 and -0.27, respectively), supporting a 2006 tagging study that associated plasma ionic imbalances with coastal mortality (19) and salinity challenge experiments that revealed higher mortality for sockeye held in saltwater as compared with isosmotic or fresh water (20). As a result, we spec-

ulate that osmoregulatory dysfunction of salmon containing the mortality-related signature may have contributed to ocean mortality and possibly stimulated faster entry into fresh water.

This combination of watershed-scale biotelemetry and functional genomics of wild salmon in nature has yielded new insight into one potential physiological mechanism associated with survivorship during return migration. Migrating salmon are expected to markedly transform gene expression, given the required physiological demands associated with upstream swimming, environmental shifts, maturation, fuel depletion, and senescence. Our study revealed a mechanistic signature associated with premature mortality of salmon measurable >1 month to <1 week ahead of death and throughout the river. Our hypothesis is that the genomic signal associated with elevated mortality is in response to a virus infecting fish before river entry and that persists to the spawning areas.

References and Notes

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Supporting Online Material
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Materials and Methods

Supplemental Results
Figs. S1 to S5
Tables S1 to S4

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The Structure of Human 5-Lipoxygenase

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The synthesis of both proinflammatory leukotrienes and anti-inflammatory lipoxins requires the enzyme 5-lipoxygenase (5-LOX). 5-LOX activity is short-lived, apparently in part because of an intrinsic instability of the enzyme. We identified a 5-LOX-specific destabilizing sequence that is involved in orienting the carboxyl terminus, which binds the catalytic iron. Here, we report the crystal structure at 2.4 angstrom resolution of human 5-LOX stabilized by replacement of this sequence.

Leukotrienes and lipoxins are potent mediators of the inflammatory response derived from arachidonic acid (AA). When leukocytes are activated, AA is released from the nuclear membrane by the action of cytosolic phospholipase A₂ and binds 5-lipoxygenase-activating protein (FLAP). The increased Ca^{2+} concentration of the activated cells simultaneously promotes translocation of 5-LOX to the nuclear membrane, where it acquires its substrate from FLAP (1, 2). AA is converted to leukotriene A₄ in a two-step reaction that produces the 5*S*- isomer of hydroperoxyicosatetraenoic acid (5*S*-HPETE) as an intermediate (3, 4).

Autoinactivation of 5-LOX activity has been described, and this loss of activity is perhaps important in limiting the synthesis of its pro- and anti-inflammatory products (5). Previous reports indicate that non-turnover-based inactivation is a consequence of an O₂ sensitivity linked to the oxidation state of the catalytic iron (6). However, not all LOXs display this hypersensitivity to O₂. For example, 8*R*-LOX activity is stable despite a solvent-exposed iron coordination sphere equivalent to that in 5-LOX (7). In similar conditions, 50% of 5-LOX activity is lost in 10 hours (8). We reasoned that 5-LOX-specific destabilizing features may confer susceptibility to non-turnover-based inactivation. Regulatory mechanisms that facilitate transient activation include targeted degradation, phosphorylation, and allosteric control of enzyme activities. Autoinactivation as a consequence of intrinsic protein instability may play a similar role. For example, the instability of the tumor suppressor protein p53, relative to its ortho-

logs such as p73, has been proposed to have a functional role (9).

On the basis of the crystal structures of two AA-metabolizing lipoxygenases [an 8*R*-LOX

from *Plexaura homomalla* (7, 10) and a 15-LOX from rabbit reticulocyte (11, 12)], each with ~40% sequence identity to 5-LOX, we identified a 5-LOX-specific lysine-rich region near the C terminus of the enzyme that might confer instability (13). In the 8*R*- and 15-LOX structures, a turn centered on amino acid 655 (5-LOX numbering) leads from the C-terminal helix to the C-terminal segment and allows the terminal carboxylate to penetrate the LOX body and bind the catalytic iron (Fig. 1, A and B). In most LOXs, amino acid 655 is a highly conserved Leu, with its side chain pointing toward an invariant Arg (Arg⁶⁵¹). A striking 5-LOX-specific feature is Lys in place of Leu at this position as part of a di- or tri-Lys peptide (fig. S1). Although numerous salt links anchor the C-terminal helix to the

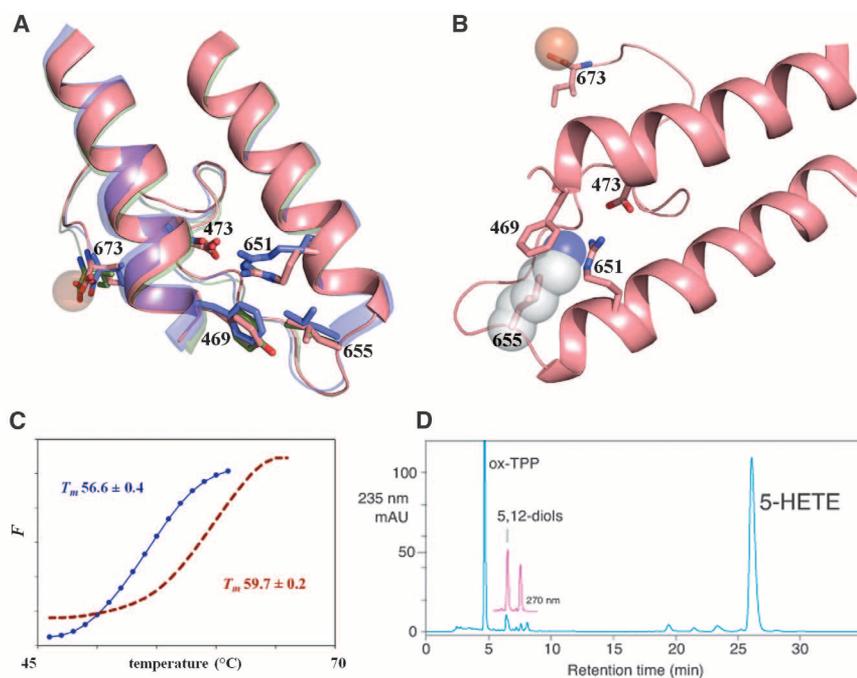


Fig. 1. Stabilization of human 5-LOX. **(A)** Superposition of the C-terminal regions of the structures of 15-, 8*R*-, and Stable-5-LOX. The C-terminal segment that leads to the catalytic Fe emanates from the helix that terminates at amino acid 655 (5-LOX numbering; Stable-5-LOX, pink; 8*R*-LOX green; 15-LOX, blue). Highly conserved amino acids (Leu and Phe/Tyr) and an invariant salt link (Asp-Arg) are depicted in stick rendering. **(B)** Detail of the turn at the end of the terminal helix. The 5-LOX-specific Lys (replaced in Stable-5-LOX with Leu) is modeled at position 655 as its most common rotamer (transparent sphere rendering). As positioned, it would interfere with the invariant salt-link and cation- π interactions. All figures were generated with Pymol (31). **(C)** Thermal denaturation of Stable-5-LOX (red) and the parent enzyme Sol-5-LOX (blue). Fluorescence (F) is monitored as a function of temperature. T_m (with SD) 56.6°C ($\pm 0.4^\circ$) and 59.7°C ($\pm 0.2^\circ$) for Sol-5-LOX and Stable-5-LOX, respectively. **(D)** High-performance liquid chromatography chromatogram. Product analysis of Stable-5-LOX reveals both 5-HETE (5-HPETE reduced by the addition of triphenylphosphine, TPP) and leukotriene A₄ hydrolysis products (5,12-diols).

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